Vol. XL No. 6

June, 1951

SCIENTIFIC EDITION

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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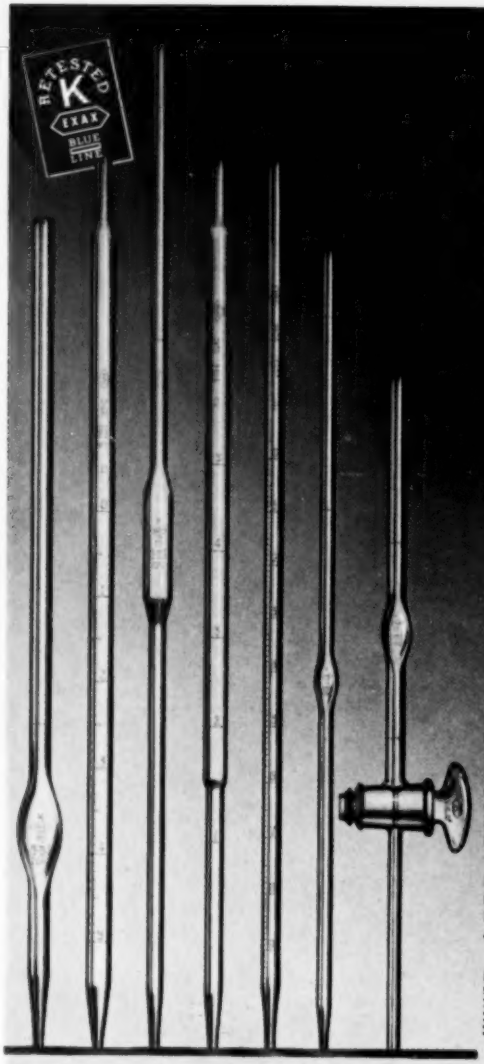

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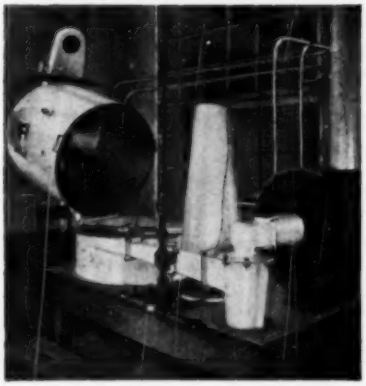

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XL — *JUNE, 1951* — NUMBER 6 — CONSECUTIVE NO. 12

Chronic Toxicity Studies on Methylcellulose in Rats*

By ROBERT O. BAUER† and A. J. LEHMAN‡,§

A standard diet of 5 per cent methylcellulose fed to rats for thirty-two weeks (averaging 690–775 mg./Kg./day/rat during this period) has no deleterious effect on growth, and significantly increased dietary intake. Also, it neither inhibited reproduction nor left evidence of pathologic change in the tissue. Fifty per cent of the diet replaced by methylcellulose significantly depresses growth through nutritional want. Methylcellulose is not hydrolyzed to cellulose and methanol in the intestinal tract of rats to any detectable extent. Methylcellulose *per se* does not appear to be absorbed from the intestinal tract of rats.

THE RECENT application of derivatives of methylcellulose as laxatives in clinical medications suggested that it would be worth while to publish chronic toxicity data obtained eight years ago. The purposes of this study were to determine any alteration of growth, food intake, fecal output, urinary formic acid production, or reproduction produced by feeding various amounts of methylcellulose to albino rats.

METHOD

Chronic Oral Toxicity.—Young albino rats of the Sprague-Dawley strain, thirty-five to forty days old and weighing 55–84 Gm., were divided into three groups of ten (five males and five females) and fed diets containing, respectively: (*a*) 5% methylcellulose (high MC diet); (*b*) 0.17% methylcellulose (low MC diet) (changed on the forty-second day of the experiment to 0.5% methylcellulose); and (*c*) no added ingredient (standard diet). The experimental diet was prepared by mixing an aqueous solution of high viscosity methylcellulose (1) with Steenbock's diet (2), spreading the resulting mixture on metal sheets, drying at room temperature, and grinding to a fine powder. The rats were segregated according to sex. They were kept in cages containing groups of five, with water and food *ad libitum*. Group body weights, and food and water consumption were recorded weekly. After about eight months on the experimental diet, the rats were mated according to the method described by Farris (3). The young of the f_1,f_2, generations were divided as above, placed on 5% methylcellulose diet, and later mated in the same manner as the f_0 generation. Representative animals from each group of the first generation were sacrificed at eight months of age for macroscopic and microscopic examination.

In the second experiment, nine young Sprague-Dawley rats, litter mates of the f_1 generation, divided according to sex into three males and six females and weighing between 55 and 116 Gm., were placed on a modified paired feeding regimen. This system was employed in order to determine the effect of massive dosage and the "bulk" producing effects of the questionably inert substance in contrast to one (cellulose flour) shown to be inert and to possess only a "bulk" effect when included in the diet (4). The nine animals were divided into three groups. Rat 1 in each of the three groups received, *ad libitum*, a diet of equal parts of the nutrient and of methylcellulose (50% methylcellulose

* Received October 2, 1950. Work done at the Department of Pharmacology, Wayne University School of Medicine, Detroit, Mich.
Published in preliminary form in *Federation Proc.*, **3**, 65 1944) and **4**, 112(1945).
† Present address: Department of Pharmacology, Boston University School of Medicine, Boston, Mass.
‡ Present address: Division of Pharmacology, Food and Drug Administration, Washington 25, D. C.
§ Acknowledgment is gratefully given to Drs. Mark E. Maun and Thomas M. Batchelor for reading the pathologic specimens.

diet). Rat 2 received, *ad libitum*, a diet of equal parts of the nutrient and cellulose flour (forming the "bulk" control), and rat 3 was given the nutrient alone (nutritive control), but in an amount equivalent to the nutrient consumed by rat 1 of the respective group the preceding day. The basic nutrient consisted of a modified Steenbock's diet as follows: 36% yellow ground corn meal, 20% linseed cake meal, 20% casein, 10% lard, 6% cod liver oil, 4% powdered alfalfa, 3% bone ash and 1% sodium chloride.

Each animal was housed in an individual metabolism cage, and daily records were kept of the urine volume, feces weight, and food and water consumption. Pooled seven-day urine samples from each rat were subjected to quantitative formic acid determinations by the method of de Eds (5).

RESULTS

The results of the chronic eight-month methylcellulose rat feedings are tabulated in Table I. These results were obtained by treating the data by the Zucker "Weight-age Relation" (6) as modified by Gray and Addis (7). These workers demonstrated that if one plots the log of the weight in grams ($\log_{10} W$) against the reciprocal of age in days ($1/d$), a straight line is obtained and k is the slope of this line which characterizes the normal rate of growth [$k = (\log W_2 - \log W_1)/(1/d_1 - 1/d_2)$]. Their formula for the straight line is: $\log_{10} W = -k(1/d) + \log A$, where A is the body weight approached asymptotically during growth. The rat continues to grow asymptotically until death, chiefly due to unclosed epiphyses. If external variables are superimposed on the normal growth pattern so as to depress growth, the k and A values become smaller. The k and A values of either sex fed 5% or less of methylcellulose do not differ significantly from their respective controls. The k and A values were considered significantly different if experimentals varied from controls by more than two standard deviations (7).

Both experimental groups ate significantly more food than did the controls. The same general trend held for succeeding generations. The final average intake of methylcellulose for males and females, respectively, was 690 and 775 mg./Kg./day given the high MC diet, and 61 and 57 mg./Kg./day given the low MC diet (see Table II).

TABLE II.—AVERAGE DAILY FOOD CONSUMPTION PER RAT FED METHYLCELLULOSE

Group of 5 Animals	Sex	F_0 Generation Mean and S. D.[a]	p Value
Control	F	8.0 ± 1.1	...
Low MC diet	F	11.4 ± 2.0	<0.02
High MC diet	F	15.5 ± 6.3	<0.01
Control	M	10.5 ± 3.2	...
Low MC diet	M	12.2 ± 1.5	<0.02
High MC diet	M	13.8 ± 3.3	<0.01

[a] Mean and Standard Deviation in Gm.

All three generations of mated rats produced litters of 8 to 11 vigorous, healthy pups. All animals were weaned at one month of age, by which time each had reached a weight of 55–60 Gm. The animals of the f_1 and f_2 generation, following weaning, were fed the high MC diet for four months. The animals of the f_1 generation grew at a rate comparable to that of the f_0 generation when fed this diet (Table I).

Macroscopic and microscopic pathologic examination of representative animals of each group of the first generation revealed essentially normal tissues. Deposition of abnormal material in the tissues was not observed.

In order to obtain still higher intake of methylcellulose, the paired feeding experiments were conducted.

Table III presents the results of this experiment. It is clear that animals on *ad libitum* intake of nutrient diet grew much more than did animals on 50% methylcellulose. However, animals on restricted diet (nutrient limited to that taken by the methylcellulose group) grew indistinguishably from: (*a*) the test animals, or (*b*) the cellulose flour group. There is no statistical difference between nutrient intake of the three groups during the test period.

At the end of ninety days the three special diets were terminated. The animals were placed on standard diet *ad libitum* for forty additional days. The k values, during this time, increased fivefold and A exceeded the control value (Table IV). Figure 1 demonstrates the plotted data for the male

TABLE I.—WEIGHT-GROWTH SLOPES AND ASYMPTOTES OF METHYLCELLULOSE-FED RATS

Groups of 5 Animals	Weight, Gm. Start	Weight, Gm. Finish	Age, Days Start	Age, Days Finish	k	A
			F_0 Generation Females			
Controls	76	183	39	272	17.2	210
High MC diet	76	212	39	238	20.6	256
Low MC diet	79	211	40	226	20.3	254
			F_1 Generation Females			
Control diet	55	170	36	163	22.6	234
High MC diet	55	200	36	163	25.9	292
			F_0 Generation Males			
Control diet	56	258	33	210	30.0	572
High MC diet	60	310	35	176	31.1	461
Low MC diet	84	291	41	267	26.7	379
			F_1 Generation Males			
Controls	55	258	36	163	31.0	428
High MC diet	55	305	37	163	34.4	496

k = Slope of growth curve.
A = Estimated asymptote or limit approached by body weight in Gm.

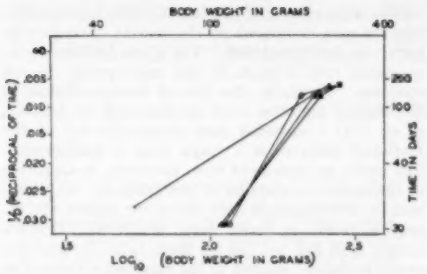

Fig. 1.—Male weight-growth curves methylcellulose paired feedings.

Control ●——●, nutritive control ⊙——⊙, 50% methylcellulose ○——○, 50% cellulose flour ▲——▲, paired rats continued on standard diet ■——■. The greater the slope, small k value, the greater is the growth depression, and, likewise, a resulting small A value at infinity.

animals given in Tables III and IV. It is concluded that the weight-growth curve depression of the bulk-containing diet is the result of nutritional want, and not the result of specific toxicity of the methylcellulose or cellulose flour.

The fecal weights from the animals whose daily diet consisted of 50% cellulose flour were 60 ± 2% of their dietary intake, whereas the fecal weights of the animals fed 50% methylcellulose were significantly higher (p = <0.02) than the former, being 73 ± 5% of the respective dietary intake. The fecal texture of the cellulose flour-fed animals was hard and dry, whereas those animals fed methylcellulose produced fecal pellets which were larger than average, soft and translucent. This translucent character is observed in highly concentrated "solutions of methylcellulose." The control rats' feces were scanty, soft, and mushy. The daily weights of the latter's feces ranged from 0.1 to 1.5 Gm. with a mean of 1.16 ± 0.23 Gm., in contrast to the 7.9 ± 1.82 Gm. average of the experimentals, or 7.9 ± 1.85 Gm. average of the cellulose flour rats' feces. It is apparent from the foregoing that the bulk-producing portions of the nutritive control diet had been reduced to a minimum, and this diet was essentially all nutritionally available to the rat.

The urinary output of the methylcellulose (3.1 ± 0.8 cc.) and cellulose flour (4.2 ± 0.8 cc.) rats was about 50% that of the nutritive controls (7.3 ± 2.1 cc.). The lower average renal fluid excretion of the experimental group probably reflects the amount of fluid excreted with the bulk of the feces, due to the hydrophilic property of methylcellulose. In contrast, the control animals required small amounts of fluid to facilitate defecation and thus eliminated most of the fluid by way of the kidneys.

The values of urinary formic acid obtained from weekly pooled urine specimens were not constant. In several specimens formic acid was not detectable. The most notable instances of the absence of formic acid in the urine were at the beginning and end of the experiment when the weather was cool. The highest weekly levels were obtained during the periods of hot, humid weather. The methylcellulose rats were less consistent in their production of formic acid than the other groups, having detectable levels in only eight out of thirteen weeks during which the experiment was conducted. The cellulose flour group of animals was the most consistent in their production of formic acid and showed no sex difference (see Table V).

In spite of the irregularity of formic acid detection of the urine, the differences between means ($\bar{x}$)

TABLE V.—PAIRED RAT URINARY FORMIC ACID LEVELS

Rat Group	Formic Acid[a]	p Value
Female controls	3.71 ± 2.80	...
Female 50% cellulose flour	2.07 ± 1.73	0.05
Female 50% methylcellulose	1.84 ± 1.24	0.02
Male control	0.56 ± 0.46	...
Male 50% cellulose flour	2.19 ± 2.60	0.01
Male 50% methylcellulose	0.81 ± 0.86	0.5

[a] Mean and standard deviation in mg. of thirteen weekly observations.

TABLE III.—WEIGHT-GROWTH SLOPES AND ASYMPTOTES OF PAIRED METHYLCELLULOSE FEEDINGS

	No.	Weight, Gm. Start	Weight, Gm. Finish	Age, Days Start	Age, Days Finish	k	A
Female Groups:							
Control	5	55	170	36	163	22.6	234
Nutritive control	2	95	155	36	131	10.3	184
50% Cellulose flour	2	95	164	36	131	11.7	201
50% Methylcellulose	2	76	131	36	131	12.6	164
Male Groups:							
Control	5	55	258	36	163	31.0	428
Nutritive control	1	113	229	32	127	13.0	290
50% Cellulose flour	1	110	240	32	127	14.4	312
50% Methylcellulose	1	116	205	32	127	10.5	249

TABLE IV.—PAIRED METHYLCELLULOSE RATS RETURNED TO STANDARD DIET

	No.	Weight, Gm. Start	Weight, Gm. Finish	Age, Days Start	Age, Days Finish	k	A
Males	6	149	191	131	171	58.95	422.5
Females	3	224	280	127	167	50.29	559.6

as well as paired data were in two instances significant. The female 50% methylcellulose rats had a mean detectable level about one-half that of the female controls, whereas there was no significant difference between the male formic acid values of these two groups. The male 50% cellulose flour animals produced about four times more formic acid than did the male controls. No difference could be found between the male control and the male 50% methylcellulose rats.

Mated females of the paired feeding experiment produced litters of 6 to 12 healthy young pups which were fed Steenbock's diet containing 5% methylcellulose for the four months following weaning. No abnormalities were noted in the animals' growth.

DISCUSSION

Deichmann, *et al.* (8), using the oral route have reported methylcellulose harmless to albino rats when given 440 mg./animal/day for eight months. Although the chronic-fed rats of this experiment consumed about one-third as much methylcellulose as Deichmann's animals, the dosage was continued in the present work through three generations without evidence of impaired growth or reproduction. A recent publication by Hodge, *et al.* (9), using longer chronic oral methylcellulose feedings, bears out this contention. Pathologic examination failed to reveal deposition of the material in body tissues. By contrast, Hueper, *et al.* (10), reported that intravenous administration of 5% solutions led to retention and deposition in various organs. Therefore, it is unlikely that the methylcellulose in these experiments was absorbed from the gastrointestinal tract.

On the basis of urinary formic acid studies, evidence was sought to show that methylcellulose was degraded in the intestinal tract. Conclusive evidence was not obtained. In our experiments, formic acid appeared in urines of both control and methylcellulose-fed animals. It seems likely that the appearance of formic acid in the urine was due to substances other than methylcellulose. In addition, the inconsistency of detection of formic acid does not permit sound conclusions as to the source of this substance. Both methanol and formic acid have been found in the urine following the ingestion of small amounts of methanol arising from the hydrolysis of the methyl esters of pectins present in many common foods (11, 12). Components of Steenbock's diet have small amounts of pectins as part of their composition. The highest urinary formic acid levels were detected during the hot, humid weather. Increased bacterial activity of intestinal flora acting on the pectins with production of free methanol could explain the elevated formic acid values during the hot weather. It is conceivable that absorption of the formic acid precursor, methanol, by the colloidal substance methylcellulose could block the absorption of methanol by the digestive tract. The significantly lower mean urinary formic acid values of the female 50% methylcellulose-fed rats compared to the female controls support this interpretation. The data for the corresponding two animals in the male group do not conform. Similarly, the fate of methylcellulose in the human digestive tract as discussed by Machle, *et al.* (13), concluded that practically all of the methoxyl ingested in a single dose of methylcellulose could be recovered from the feces, irrespective of the nature of the diet of the subject. On a normal or low-roughage diet the agent passes the digestive tract, in large part, unaltered. Urinary formic acid did not rise in these experiments after methylcellulose was fed to the rats, but remained at levels less than or the same as the control animals.

Individual differences, both as to sex and variety, in the formation and excretion of formic acid apparently depend in part on the individual's ability to detoxify methanol by other routes than catabolism to formic acid. The variability is well illustrated by the sex differences of the paired feedings.

CONCLUSIONS

Methylcellulose, fed to rats over three generations in quantities amounting to slightly less than 5.0 and 0.5 Gm. per Kg. body weight, has no deleterious effect on growth, and significantly increased dietary intake. Also, it neither inhibits reproduction nor leaves evidence of pathologic changes in the tissues. Fifty per cent of the diet replaced by methylcellulose significantly depresses growth through nutritional want.

Methylcellulose is not hydrolyzed to cellulose and methanol in the intestinal tract of rats to any detectable extent.

Methylcellulose *per se* does not appear to be absorbed from the intestinal tract in rats.

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Dextro- and *Levo*-N-Methyl-1,2-Diphenyl-2-Hydroxyethylamine Salts of Benzylpenicillin*

By VERNON V. YOUNG†

A series of convenient reactions for the preparation of *d*- and *l*-N-methyl-1,2-diphenyl-2-hydroxyethylamine salts of benzylpenicillin, and a concurrent method for the resolution of racemic N-methyl-1,2-diphenyl-2-hydroxyethylamine are described.

FOR SOME time, this laboratory has been engaged in preparing crystalline organic salts of benzylpenicillin which are only slightly water soluble. A number of such salts have been prepared from alkyl- and aryl-substituted ethanolamines. This paper deals with the preparation of *d*- and *l*-N-methyl-1,2-diphenyl-2-hydroxyethylamine salts of benzylpenicillin, as well as the isolation and characterization of *d*- and *l*-N-methyl-1,2-diphenyl-2-hydroxyethylamine. The penicillin salts are white, needle-like crystalline compounds whose microbiological activity approaches the theoretical potency of 1,058 units per mg.

l-N-Methyl-1,2-diphenyl-2-hydroxyethylamine salt of benzylpenicillin was obtained in excellent yield by reacting aqueous solutions of potassium benzylpenicillinate and racemic N-methyl-1,2-diphenyl-2-hydroxyethylamine hydrochloride in a molar ratio of 1:2. The precipitated benzylpenicillin salt was isolated by filtration and *d*-N-methyl-1,2-diphenyl-2-hydroxyethylamine was recovered from the filtrate by adjusting the *p*H. *d*-N-Methyl-1,2-diphenyl-2-hydroxyethylamine salt of benzylpenicillin was obtained by reacting butyl acetate solutions of *d*-N-methyl-1,2-diphenyl-2-hydroxyethylamine and free benzylpenicillinic acid. *l*-N-Methyl-1,2-diphenyl-2-hydroxyethylamine was obtained by acid decomposition of a water-ether suspension of the respective penicillin salt.

It is considered worth while to record the preparations of these penicillin salts, not only because of their possible application as repository forms of benzylpenicillin, but also because their preparation provides a simple method for the resolution of racemic N-methyl-1,2-diphenyl-2-hydroxyethylamine. It is also of interest to note that although N-methyl-1,2-diphenyl-2-hydroxyethylamine contains two asymmetric carbon atoms and thus four optical isomers and two racemic mixtures are possible, only one racemate and two optically active isomers were isolated in these experiments from the compound obtained by the reductive amination of benzoin with monomethylamine. These findings are in general agreement with those advanced by Read and Steele (1), and later by McPhee and Erickerson (2), who found that in the case of 1,2-diphenyl-2-hydroxyethylamine, predominantly one racemate was obtained, depending on whether the compound was prepared by the reduction of benzoinoxime or by the Erlenmeyer (3) reaction.

In Table I are tabulated some of the physical properties, as well as analyses, of the compounds prepared and isolated in the following experiments.

Experimental and clinical data on the duration of penicillin blood levels and tolerance in benzylpenicillin-hypersensitive patients with these benzylpenicillin salts will be published elsewhere.

EXPERIMENTAL

Racemic N-Methyl-1,2-diphenyl-2-hydroxyethylamine.—The procedure used was a modification of that employed by Skita and Keil (4) and Morey (5). A mixture of 53 Gm. (0.25 mole) of synthetic benzoin, 10 Gm. of anhydrous monomethylamine, 10 Gm. of Raney nickel, and 400 cc. of methanol was shaken, heated to 100° (brought to desired temperature within thirty to forty-five minutes), and hydrogenated at 100° and 1,000 lb. pressure. After the reduction was complete (two to three hours), the catalyst was removed by filtration and the filtrate evaporated to dryness under reduced pressure. The residue was recrystallized from hot toluene. The recrystallized material was obtained as white needle-like crystals.

The hydrochloride was obtained by saturating an acetone or ether solution of the free base with dry hydrogen chloride, and was recrystallized from a methanol-acetone mixture.

***l*-N-Methyl-1,2-diphenyl-2-hydroxyethylamine Salt of Benzylpenicillin.**—A solution of 14.0 Gm. (0.0375 mole) of potassium benzylpenicillinate in 50 cc. of water was added rapidly to a solution of 19.7 Gm. (0.075 mole) of racemic N-methyl-1,2-diphenyl-2-hydroxyethylamine hydrochloride in 800 cc. of water. After standing several hours, the precipitated crystalline penicillin salt was collected on a filter, rinsed with 50 cc. of water, and dried; yield was 18.6 Gm. (88.3%). Recrystallization of 10 Gm. of the crude product from a warm methanol-ether solution (1:3) gave 7.0 Gm.

* Received February 24, 1951, from the Pharmaceutical Research Division, Commercial Solvents Corporation, Terre Haute, Ind.

† The author is indebted to Dr. F. H. Schultz, Jr., and Dr. H. E. Stavely for helpful suggestions throughout this work.

TABLE I.—PHYSICAL AND ANALYTICAL DATA

Compound	Empirical Formula	M. P., °C. (Uncorr.)	$[\alpha]_D^{25}$	Calcd.			Found			Penicillin Potency[d] u./Mg.
				C	H	N	C	H	N	
Racemic N-methyl-1,2-diphenyl-2-hydroxyethylamine	$C_{15}H_{17}NO$	136.0–137.0[b]	.....	79.25	7.53	6.16	79.35	7.41	5.95	...
Racemic N-methyl-1,2-diphenyl-2-hydroxyethylamine hydrochloride	$C_{15}H_{18}NOCl$	270.0–271.0, subl.	.....	68.30	6.87	5.31	68.47	6.88	5.16	...
l-N-methyl-1,2-diphenyl-2-hydroxyethylamine salt of benzylpenicillin	$C_{31}H_{34}N_2O_5S$	186.0–188.0, dec.	+112.5°[c]	66.28	6.28	7.48	66.33	6.18	7.38	1,050
l-N-methyl-1,2-diphenyl-2-hydroxyethylamine	$C_{15}H_{17}NO$	131.0–131.5	−40.0°[c]	79.25	7.53	6.16	79.54	7.40	6.06	...
l-N-methyl-1,2-diphenyl-2-hydroxyethylamine hydrochloride	$C_{15}H_{18}NOCl$	297.0–297.5, subl.	−80.6°[c]	69.30	6.87	5.31	68.42	6.73	5.10	...
d-N-methyl-1,2-diphenyl-2-hydroxyethylamine salt of benzylpenicillin	$C_{31}H_{34}N_2O_5S$	148.0–151.0, dec.	+234.0°[c]	66.28	6.28	7.48	66.21	6.18	7.33	1,073
d-N-methyl-1,2-diphenyl-2-hydroxyethylamine	$C_{15}H_{17}NO$	129.0–131.0	+38.2°[c]	79.25	7.53	6.16	79.02	7.37	5.89	...
d-N-methyl-1,2-diphenyl-2-hydroxyethylamine hydrochloride	$C_{15}H_{18}NOCl$	295.0–296.0, subl.	+78.7°[c]	68.30	6.87	5.31	68.77	6.79	5.16	...

[a] The author is indebted to Mr. C. Carr of the Analytical Division of these Laboratories for all analyses.
[b] A. Skita and F. Keil report m. p. 134° for the free base and 250° for its hydrochloride.
[c] C = 1 (in methanol).
[d] Activity against *Staph. Aureus*; sodium benzylpenicillinate standard, 1,667 u./mg.

***l*-N-Methyl-1,2-diphenyl-2-hydroxyethylamine.**—A suspension of 16.8 Gm. (0.03 mole) of *l*-N-methyl 1,2-diphenyl-2-hydroxyethylamine salt of benzylpenicillin in 250 cc. of ethyl ether and 750 cc. of water was stirred mechanically and the *p*H adjusted to 1.0 with 10% hydrochloric acid. The water layer was removed and made alkaline to *p*H 9.0 with 10% sodium hydroxide solution. The precipitated base was collected on a filter, rinsed with water, and dried; yield was 6.0 Gm. (88.2%). The crude product was recrystallized from ligroin (b. p. 86–100°) yielding needle-like crystals.

The hydrochloride was obtained by saturating an acetone or ether solution of the free base with dry hydrogen chloride, and was crystallized from methanol-acetone mixture.

***d*-N-Methyl-1,2-diphenyl-2-hydroxyethylamine.**—The filtrate from the preparation of *l*-N-methyl-1,2-diphenyl-2-hydroxyethylamine salt of benzylpenicillin was adjusted to *p*H 9.0 with 10% sodium hydroxide solution. The precipitated base was collected on a filter, rinsed with distilled water, and dried; yield was 8.1 Gm. (95.3%). Recrystallization of the crude material from ligroin (b. p. 86–100°) gave long needle-like crystals.

The hydrochloride was obtained by saturating an acetone or ether solution of the free base with dry hydrogen chloride and was recrystallized from a methanol-acetone mixture.

***d*-N-Methyl-1,2-diphenyl-2-hydroxyethylamine Salt of Benzylpenicillin.**—An equivalent amount of free benzylpenicillinic acid extracted into 700 cc. of butyl acetate was added to a solution of 22.7 Gm. (0.1 mole) of *d*-N-methyl-1,2-diphenyl-2-hydroxyethylamine dissolved in 1,000 cc. of butyl acetate. Crystallization was induced by scratching or seeding. The reaction mixture was stored for eighteen to twenty-four hours at 0–5°. The crystals were collected on a filter, rinsed with a small amount of butyl acetate, and dried; yield was 37.8 Gm. (67%).

SUMMARY

A series of convenient reactions for the preparation of *d*- and *l*-N-methyl-1,2-diphenyl-2-hydroxyethylamine salts of benzylpenicillin is described. Concurrently, the racemic amine is resolved into its *dextro* and *levo* forms by use of benzylpenicillinic acid.

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Toxicity Studies on Alkyldimethylbenzylammonium Chloride in Rats and in Dogs*

By B. V. ALFREDSON,† J. R. STIEFEL,† FRANK THORP, JR.,‡ W. D. BATEN§, and M. L. GRAY‡

Results are presented concerning the effects of alkyldimethylbenzylammonium chloride when fed in the diets of rats and dogs and when given in single doses by stomach tube to rats. Rats were fed for a two-year period in one experiment and for four months in another. Dogs were fed the test agent for fifteen weeks. The results of these studies show no demonstrable evidence of toxicity for alkyldimethylbenzylammonium chloride when fed in concentrations of 0.25 per cent or less in the diet of the rats and 0.12 per cent or less in the diet of the dogs. The acute LD_{50} of this compound in rats, as determined by single oral dosages, was found to be 234.3 ± 26.5 mg./Kg. body weight.

STUDIES on the toxicity of the quarternary germicides have lagged far behind their industrial development and use. Alkyldimethylbenzylammonium chloride is no exception to this rule. Fitzhugh and Nelson (1) have reported, in a long-term chronic oral toxicity study, that alkyldimethylbenzylammonium chloride gave evidences of toxicity to weanling albino rats at concentrations of 0.063 per cent or more in the diet, and at a concentration of 0.5 per cent all the animals died within ten weeks. In an investigation of four quaternary ammonium compounds, Shelanski (2) administered (as one of the agents studied) alkyldimethylbenzylammonium chloride (as commercial Onyx BTC) by stomach tube, daily amounts of 25, 12.5, and 5 mg./Kg., respectively, to three groups of rats over a period of two years. He noted that the animals at the 25 mg./Kg. test level began to show a progressive decrease in body weight beginning at fifteen months and continuing for the remainder of the animal's life. In a short-term study, Harshbarger (3) fed alkyldimethylbenzylammonium chloride (as commercial Zephiran) to white rats for a few weeks and found that 0.3 per cent in the diet produced no difference in body weight gains. Toxicity to other animal species and by other routes of administration is considered in the reviews of Woodard and Calvery (4) and Lawrence (5).

The only information as to the magnitude of the single toxic oral dose of alkyldimethylbenzylammonium chloride in the rat which we have been able to find is included in the reports by Woodard and Calvery (4) and by Shelanski (2). The former authors state the approximate LD_{50} to be of the order of 350 mg./Kg., while Shelanski reports this value to be 445 mg./Kg.

The purpose of this report is to add to the foregoing data our findings concerning the acute and chronic oral toxicity of alkyldimethylbenzylammonium chloride to the albino rat, and to present our observations concerning the toxicity of this compound to dogs.[1]

PROCEDURE

Chronic Toxicity Experiments on Rats.—Albino rats from a stock maintained in the Michigan State College Chemistry Department for many years were placed on the experiments at twenty-one days of age. Two experiments were run: one subacute pilot experiment (to yield information concerning dosage levels), and one larger chronic toxicity experiment. In the pilot experiment, 66 rats divided into eight groups of at least five animals each were allowed free access to a diet consisting of ground Purina Laboratory Chow with added alkyldimethylbenzylammonium chloride[2] for twelve weeks. Dietary levels of 1.0, 0.5, 0.25, 0.125, 0.062, 0.031, 0.015, and 0.000% of the test agent were used. All of the animals survived the trial period, with the exception of the five rats on the 1% level and one rat on the 0.5% level.

The two-year experiment was set up on 24 litters of seven animals each, distributed in a randomized block design employing all the dose levels of the

* Received November 21, 1950, from Michigan State College, East Lansing, Mich.
Supported in part by a grant-in-aid from Sterwin Chemicals, Inc., New York, N. Y. Published with the approval of the Director of Michigan Agricultural Experiment Station as Journal Series No. 1,192.
† Department of Physiology and Pharmacology, School of Science and Arts and School of Veterinary Medicine, Michigan State College.
‡ Section of Animal Pathology, Michigan Agricultural Experiment Station, East Lansing, Mich.
§ Department of Mathematics, Michigan State College, and statistician for Michigan Agricultural Experiment Station, East Lansing, Mich.

[1] The authors wish to express their appreciation to the Chief and the Staff of the Division of Pharmacology of the Food and Drug Administration for their many helpful suggestions during the course of this investigation.
[2] The alkyldimethylbenzylammonium chloride used in the experiments reported here was from one lot supplied by Sterwin Chemicals, Inc., in the form of Roccal, a commercial aqueous solution. This lot contained 9.77% alkyldimethylbensylammonium chloride.

pilot experiment except the highest (1.0%) level. The distribution was such that one rat from each litter was placed on each of the seven dose levels. The sexes were distributed throughout the block so that equal numbers of each sex (i. e., 12 males and 12 females) were placed on each treatment. Each rat was kept in an individual cage in a room maintained at 75 ± 2° F. Body weights and food consumption were determined at weekly intervals.

Blood studies included determinations of the total red and white cell counts, differential white cell count, and the determination of hemoglobin concentration by a modified Sanford method (6). Blood determinations were made at bimonthly intervals in 10 out of the 24 litters on each level, including controls.

All animals, including those dying spontaneously during the experiment, were autopsied and examined for gross pathological changes. In all cases, specimens were preserved routinely from the following tissues: heart, spleen, kidney, urinary bladder, bone (femur), bone marrow (femur), stomach, duodenum, jejunum, ileum, cecum, rectum, liver, and adrenal gland. Those obtained from the 10 litters on which blood studies were conducted and those tissues from animals from the other litters which presented gross evidence of abnormality on autopsy were sectioned and stained with hemotoxylin and eosin (7) and examined microscopically[3] for evidence of any pathological change. A number of tissues were also fixed in formol-saline, frozen with carbon dioxide, sectioned, and stained for fat with Sudan IV (7).

Experiments on Dogs.—Seven healthy, mature, mongrel pound dogs which had attained full growth and which had been maintained in our dog quarters on a normal diet (ground Dickinson dog food nuggets[4]) for from six to twelve months were used in this experiment. Two dogs were fed alkyldimethylbenzylammonium chloride in this diet at a level of 1%. A single dog was placed in each of the following levels: 0.5, 0.25, 0.125, 0.062, and 0.031%, respectively. Each animal was gradually brought up to test level over a period of two weeks and maintained on its level for fifteen weeks. The dogs were weighed at weekly intervals and observed daily for clinical symptoms. While it was not possible to keep accurate records for food consumption, observations were made as to whether this was apparently normal, fair, or poor.

Since a different diet was used for the dogs than for the rats, a small parallel experiment was conducted on rats using one male and one female on each of the dietary levels fed to the dogs. At the end of the experimental period, all of the animals were sacrificed and examined for any gross evidence of pathologic change. Tissue specimens taken from the dogs included the left ventricle, spleen, kidney, urinary bladder, stomach, duodenum, jejunum ileum, cecum, rectum, and liver.

Acute Toxicity Experiments on Rats.—Alkyldimethylbenzylammonium chloride was administered by stomach tube to groups of eight male and eight female rats at each of seven dosage levels, progressing logarithmically in steps of one-tenth of the log unit between the logarithm of 79.4 and 316.2 mg./-Kg. body weight, inclusive. All treatments were given in the afternoon and all animals were fasted from 5:00 p. m. on the preceding day (approximately eighteen to twenty hours). The distribution of the rats was such that litter mates were represented on all levels of dosage. The litters ranged in age from one hundred and nine to two hundred and twelve days at the time of dosage.

The surviving animals were killed with ether vapor, weighed, and autopsied on the tenth day following treatment. The rats not surviving the dose of alkyldimethylbenzylammonium chloride were autopsied as soon after death as was possible. Autopsy observations were limited, in a majority of the cases, to gross inspection. However, microscopic observations were made in a few instances.

RESULTS AND DISCUSSION

Two-Year Experiment on Rats: Mortality.—The per cent mortality of the rats in the two-year chronic toxicity experiment is presented in Table I. The table shows that 50% of the 24 animals on the 0.5% level failed to survive fifty days. All but one of these died in from eight to twenty-two days. From the clinical symptoms prior to death and the autopsy picture post-mortem, it appears likely that these subjects died from the effects of alkyldimethylbenzylammonium chloride. In no other instance could death be attributed to the effects of the test agent fed in the diet. Twenty males and 35 females, or a total of 32.7% of the original number of 168 rats, survived the experiment.

Body Weight.—An analysis of variance was carried out on the gains in weight at the end of two weeks[5] and at the end of six months on all animals. At the end of twelve months, the data on body weight increments were analyzed[6] for the rats on the 0.25, 0.125, 0.062% levels and control. Non-orthogonality in these groups resulting from the death of some of the animals was corrected in this analysis by computing adjusted treatment effects, eliminating the effect of differences in levels of alkyldimethylbenzylammonium chloride. The procedure was essentially that of Yates (8) and Bose (9). An analysis known as "fitting constants to the data" (10) was employed on the gains in body weight for the entire set of data, obtained at the end of fifteen months, to determine significance between dosage means. This type of analysis was used since, due to mortality, there was no longer an equal number of males and females, and also because there were not the same number of animals on each of the dosage levels.

All of these statistical techniques revealed a significant difference between the mean of the gains of the animals on the 0.5% level and the means of the other doses, but no significant differences among the other means, including the control animals.

[2] The authors wish to acknowledge the assistance of E. T. Hallman, Professor Emeritus, Department of Animal Pathology, School of Veterinary Medicine, Michigan State College, for his invaluable assistance in the microscopic examination of the stained sections of tissues.

[3] Manufactured by the Albert Dickinson Co., Chicago, Ill

[5] E. W. Kivela, Mich. State Dept. of Health, carried out the statistical treatment of this portion of the data.

[6] Statistical treatment of this portion of the data was performed under the direction of Prof. Leo Katz of the Dept. of Mathematics, Michigan State College.

TABLE I.—PER CENT MORTALITY OF RATS: TWO-YEAR CHRONIC TOXICITY EXPERIMENT

Age, Days	Levels of Alkyldimethylbenzylammonium Chloride in the Diet							
	0.5	0.25	0.125	0.062	0.031	0.015	Control	Total
1-200	50.0[a]	0	4.2	0	12.5	0	0	9.5
201-400	0	0	0	0	4.2	4.2	8.3	2.4
401-600	4.2	25.0	25.0	25.0	16.7	20.8	29.2	20.8
601-728	25.0	25.0	37.5	41.7	37.5	41.7	33.3	34.5
Per cent surviving at termination of experiment (728 days)	20.8	50.0	33.3	33.3	29.2	33.3	29.2	32.7
Total No. rats per level	24	24	24	24	24	24	24	168

[a] All of these animals failed to survive fifty days.

This is at variance with the findings reported by Fitzhugh and Nelson (1). At the end of the first year, these investigators found the difference between gain in weight in the controls and in animals on 0.25% alkyldimethylbenzylammonium chloride to be highly significant, and the difference between the controls and those on a 0.125% level to be significant.

Food Consumption.—Correlation of total food consumption with increase in body weight carried out for the entire set of data at the end of fifteen months failed to reveal any significant difference in any of the levels below 0.5% alkyldimethylbenzylammonium chloride in the diet. The animals on the 0.5% level ate a significantly smaller amount of food than those on the other levels.

Blood Studies.—Analyses of variance conducted on all the blood data from 10 male and female rats on each dosage level below 0.5% and control at the end of thirteen months and seventeen months showed no significant differences among the means of these groups.

Pathology.—The twelve animals on the 0.5% level which died within the first fifty days of the experiment showed only the following gross pathological changes: the presence of a red- to brownish-colored viscid material in the stomach and the upper level of the small intestine, and the appearance of acute gastritis involving the glandular portion of the gastric mucosa. On microscopic examination, these structures showed a variable amount of superficial necrosis of the mucosa. No pathological changes could be discerned in the other tissues (liver, kidney, etc.) examined. These observations are in substantial agreement with the findings of Fitzhugh and Nelson (1). Similarly, the anorexia and diarrhea during life and the distention of the cecum with fluid at necropsy were the same as that described by these authors. In no case were similar changes noted in rats fed alkyldimethylbenzylammonium chloride on levels in the diet below 0.5%.

In interpreting the pathological findings in the remainder of the experimental animals, it would seem necessary not only to consider the possible effects attributable to the test agent, but also the changes which one might anticipate as being associated with the process of aging in the laboratory rat. No information of this type was available on the strain of rat used in these experiments. The outstanding necropsy findings in all animals on the chronic experiment are summarized in Table II. This table reveals the occurrence of three important pathologic changes, *viz.*, pneumonia, neoplasm, and nephritis. These changes were most notable in rats autopsied after the 400th day on the experiment. No significant differences between the rats on the various levels of the test agent including the controls are apparent in the results.

Results of Feeding Experiments on Dogs.—Of the seven dogs fed alkyldimethylbenzylammonium chloride in the diet, the four animals on the lower levels of dosage survived the fifteen-week period of the experiment. One of the animals on the 1% level was sacrificed when moribund at the end of the seventh week of the experiment; the other subject died during the twelfth week. The dog on the 0.5% level was also considered a fatality since it was moribund at the end of fifteen weeks. Figure 1 shows the weekly changes in body weight of all the dogs used in the experiment. The four animals on the levels above 0.12% exhibited a definite decrease in their body weights. While no accurate records were obtained, it was noted that food consumption in these four dogs was much less than in the case of the remaining animals. The weight changes in the three subjects on the levels below 0.25% do not appear to be significant.

TABLE II.—SUMMARY OF AUTOPSY FINDINGS IN RATS: TWO-YEAR CHRONIC TOXICITY EXPERIMENT

Necropsy Findings	Levels of Alkyldimethylbenzylammonium Chloride in the Diet, %														Totals		
	0.5		0.25		0.125		0.062		0.031		0.015		Control		Males	Females	Males and Females
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*			
Neoplasm	1	2	7	3	4	1	3	2	7	2	6	1	7	1	24	23	47
Pneumonia	2	1	3	3	7	3	8	2	6	1	4	1	5	4	28	22	50
Nephritis	0	0	1	5	3	3	6	2	2	1	4	1	4	2	22	12	34
Miscellaneous	7	0	1	0	3	0	3	0	1	0	1	0	2	0	..	..	18
No diagnosis, due to post-mortem change	8	0	1	0	0	0	0	0	1	0	0	0	0	0	..	..	10
No significant findings	1	2	0	4	2	3	1	5	2	4	2	6	1	2	..	..	35

a = Animals dying before termination of the experiment.
b = Surviving animals sacrificed at termination of the experiment.

TABLE III.—MORTALITY OF RATS FOLLOWING SINGLE ORAL DOSES OF ALKYLDIMETHYLBENZYLAMMONIUM CHLORIDE

	Sex	Dosage Level (Mg./Kg.) 316.2	251.2	199.5	158.5	125.9	100.0	79.4	Totals
No. dying	M	8	6	2	2	2	1	0	21
	F	7	4	4	0	2	0	1	18
No. surviving	M	0	2	6	6	6	7	8	35
	F	1	4	4	8	6	8	7	38

No gross or microscopic changes which could be attributed to the test agent were observed in any of the dogs on the levels below 0.5% alkyldimethylbenzylammonium chloride in the diet. The changes in the dogs on the 1% and the 0.5% levels resembled, in general, those seen in rats. The contents of the stomach and small intestine resembled partially digested blood. The glandular portion of the stomach contained three small erosions in one case and hemorrhage in the mucosa in the other. A very superficial necrosis of the mucosa of the small intestine was seen microscopically in one case. No other pathological changes were seen in any of the other tissues or organs examined. The results in the parallel experiment conducted on rats appeared to be identical with those seen in the two-year chronic toxicity experiment reported elsewhere in this paper.

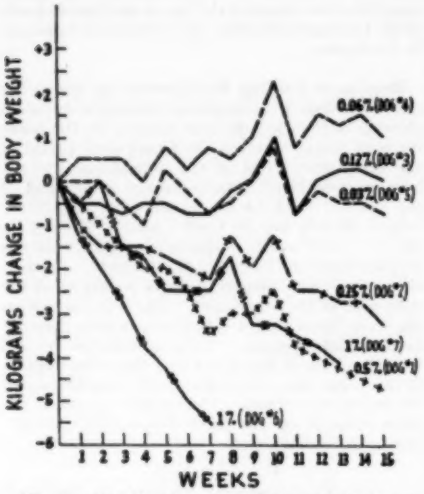

Fig. 1.—Weekly changes in body weight in mature dogs fed various levels of alkyldimethylbenzylammonium chloride in the diet.

Results of Acute Toxicity Trials in Rats: Mortality.—The mortality on the various dosage levels according to sex is shown in Table III. It will be noted that one animal survived on the highest level and one died on the lowest level of dosage. The greatest incidence of deaths of the rats as a result of treatment with alkyldimethylbenzylammonium chloride occurred in the period between the 19th and 34th hour following dosage. No litter differences attributable to age were apparent. The LD_{50} when calculated from the mortality data according to the method of Miller and Tainter (11) was 234.3 ± 26.5 mg./Kg. of body weight.

Clinical Symptoms.—The clinical symptoms manifested were principally inappetence, depression, tucked-up appearance, diarrhea, and bleeding from the nose. Various combinations of these symptoms were invariably present in the rats not surviving treatment. They were present in only a few of the surviving animals; usually disappearing in forty-eight hours.

Water consumption by the nonsurvivors was approximately the same as that of the survivors except in the case of moribund animals, when it was less. It was observed that the food consumption of the nonsurvivors was practically zero.

During the ten days following treatment, the surviving females gained weight on all the dosage levels; the males lost weight on two of the three highest levels and gained less than the females on two of the three lowest levels of dosage.

Pathology.—Gross pathologic changes in the rats not surviving the test agent consisted largely of varying grades of acute gastritis and congestion of the mucosa of the small intestine. The stomach contained varying quantities of a white curd-like material which, upon microscopic examination in one animal picked at random, was determined to be composed of coagulated secretions and necrotic epithelial cells. The small intestine was generally distended with a material of a consistency which resembled, in many instances, clotted blood plasma. The cecum, with but one exception, was greatly distended with a slightly viscid fluid. The entire alimentary tract, from the stomach to the cecum, inclusive, invariably presented a flaccid appearance.

In the surviving animals the only gross abnormality observed was erosion of portions of the gastric mucosa in five cases.

CONCLUSIONS

Alkyldimethylbenzylammonium chloride, when fed in the diet to albino rats at levels tested below 0.5 per cent, produced no demonstrable effects on growth, food consumption, the blood picture, incidence of pathological changes, or longevity, which could be construed to be in any way significantly different from the control animals. In those animals dying on the levels above 0.25 per cent, weight loss and evidence of gastroenteritis were the only changes observed which could be attributed to alkyldimethylbenzylammonium chloride.

From the results on a limited number of dogs, it would appear that alkyldimethylbenzylammonium chloride is more toxic to this species than to rats, since evidence of detrimental effect was observed in animals on the levels tested above 0.12 per cent of this agent in their diet.

The mortality data in rats, given alkyldimethylbenzylammonium chloride in single oral doses ranging from 79.4 to 316.2 mg./Kg. body weight, indicated the LD_{50} to be 234.3 ± 26.5 mg./Kg. The only pathological changes observed in this series were those of a pure gastrointestinal irritant.

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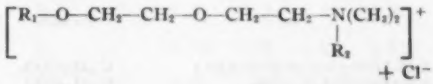
The Effect of Ring Chlorination on the Antibacterial Action of Aryloxyethoxyethylbenzyldimethylammonium Chlorides*,†

By PHILIP WEISS, M. G. CORDASCO, W. CARMAN, and L. REINER‡

A series of new quaternary compounds of the aryloxyethoxyethylbenzyldimethylammonium chloride type has been synthesized, and the effect of simultaneous alkylation of the phenoxy group and chlorination of one or both benzene rings on the antibacterial activity has been determined. The enhancing effect of chlorination is relatively greater in compounds having short alkyl substituents in the phenoxy group. If the alkyl substitutions in the phenoxy group are greater than that corresponding to eight carbon atoms, chlorination decreases rather than enhances the antibacterial activity. Chlorination of the benzyl group in the 4-position is more effective than in the 2-position. Dichlorination in the 3- and 4-position is slightly more effective than in the 2- and 4-position. Chlorination of the phenoxy group in the 6-position decreased the activity of compounds in which the 2- and 4-positions were occupied. One of the compounds tested proved to be less toxic and less irritating than one of the widely used quaternary ammonium compounds.

COMPARISON of the antibacterial properties of ring chlorinated quaternary ammonium salts with the corresponding unchlorinated compounds has resulted in controversial data. Rawlins, Sweet, and Joslyn (1) reported that the introduction of *o*-chloro or *p*-chloro group in the benzyl group of 4-(2-methyl-4,4-dimethylpentyl-2)-phenoxyethoxyethylbenzyldimethylammonium chloride did not increase the germicidal activity and may actually decrease it. Similarly, Shelton, *et al.* (2), found a decrease in activity in cetyl-*o*-chlorobenzyldimethylammonium bromide as compared with the unchlorinated compound.

In contrast to this, Dubois and Dibblee (3) and Lawrence, *et al.* (4), reported enhanced germicidal activity due to the presence of chloro groups in the benzyl group of alkyldimethylbenzylammonium chlorides; two or three chlorine atoms being more active than those containing one chlorine atom or none. Buck, Reiner, and Sherwood (5) synthesized a series of chloro-substituted quaternary ammonium compounds having the formula



where R_1 represents a haloaryl or an alkylated haloaryl group and R_2 is a halogenated benzyl group. Halogen substitution in R_1 and R_2 showed increased germicidal activity over the unchlorinated parent compound in most instances.

* Received October 6, 1950, from the Research Laboratories, Wallace and Tiernan Products, Inc., Belleville 9, N. J.
† Presented before the Division of Medicinal Chemistry, American Chemical Society, Chicago, Ill., September, 1950.
‡ The authors wish to express their appreciation to Mr. E. W. Post of our Analytical Laboratory for the analyses.

Since the activity of quaternary ammonium compounds, which are nearly linear in structure, depends on the size of the molecule, it occurred to us that the effect of ring chlorination might depend on the size of the molecule and that a maximum in germicidal activity might be reached as the size of the molecule is progressively increased. To further investigate this possibility, we prepared a series of new compounds of the aryloxyethoxyethylbenzyldimethylammonium chloride type in which R_1 contained alkyl substitutions in the benzene nuclei ranging from C_2 to C_{10} with and without chloro groups, and R_2 was the benzyl or chlorobenzyl group, and compared their antibacterial properties with the respective unchlorinated quaternary ammonium compounds.

One representative of this group, the 4-(2-methylbutyl-2)-2-chlorophenoxyethoxyethyl-4′-chlorobenzyldimethylammonium chloride, showed high bactericidal activity, and low toxicity and was therefore tested against various organisms for bacteriostatic and fungistatic activity. Chronic and acute toxicities as well as conjunctival and skin irritation studies were carried out on this compound.

EXPERIMENTAL

Alkylchlorophenols.—Chlorine gas was bubbled through a glacial acetic acid solution of the respective alkyl phenol. The compounds (Table I) were isolated as colorless oils by distillation *in vacuo*.

Substituted Phenoxyethoxyethyl Chlorides.—To freshly prepared sodium ethoxide was added the alkyl or alkylchlorophenol and the resultant solution was added dropwise to a refluxing solution of β,β′-dichlorodiethyl ether in absolute ethanol. The desired products were obtained by fractional distillation (Table II).

Substituted Phenoxyethoxyethyldimethyl Amines.—These compounds were prepared by adding the substituted phenoxyethoxyethyl chlorides to a 33.3% dimethyl amine in methanol solution and heating the mixture in a sealed tube at 125° for eight hours. The tertiary amines (Table III) were obtained by distillation *in vacuo*.

Substituted Phenoxyethoxyethylchlorobenzyldimethyl Ammonium Chlorides.—Condensation of the respective tertiary amine with chlorinated benzyl chlorides in anhydrous ether yielded the desired quaternary ammonium compounds (Table IV).

BACTERIOLOGICAL STUDIES

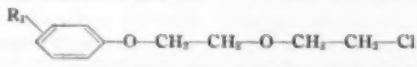
Bactericidal Action.—The bactericidal activity of the quaternary ammonium salts was determined in the following manner: a series of dilutions of the quaternary ammonium salt was prepared in distilled water using a volume of 5 cc. per tube. At a constant temperature of 20°, each tube was inoculated at thirty-second intervals with 0.5 cc. of a twenty-four hour broth culture of the test organism. Using a sterile 4-mm. platinum loop, samples were removed from each tube after vigorous shaking. Five minutes after the inoculation, they were placed in 10 cc. of sterile nutrient broth and incubated at 37°. This procedure was repeated after ten- and fifteen-minute periods of contact. A similar test was also carried out at 37°, and at both temperatures in the presence of 2% peptone.

TABLE I.—PHYSICAL AND ANALYTICAL DATA OF ALKYLCHLORO PHENOLS

Phenol	Formula	Yield	B. P. (Uncorr.), ° C.	Chlorine, % Calcd.	Chlorine, % Found
4-(2-Methylbutyl-2)-2-chloro	$C_{11}H_{15}ClO$	81	87–92/2 mm.	17.67	17.88
3,5-Dimethyl-4-chloro	C_8H_9ClO	77	[a]	22.68	22.51
4-(2-Methyl-4,4-dimethylpentyl-2)-2-chloro	$C_{14}H_{21}ClO$	80	117–124/0.2 mm.	14.58	14.66
2,4-Di(pentyl-3)-6-chloro	$C_{16}H_{25}ClO$	75	128–132/2.5 mm.	13.06	13.05
4-(2-Methylbutyl-2)-2,6-dichloro	$C_{11}H_{14}Cl_2O$	50	113–116/0.25 mm.	30.73	30.70

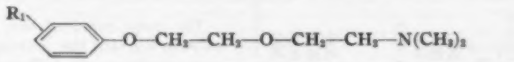
[a] Melting point 115–116°: prepared by reacting 3,5-dimethylphenol with sulfuryl chloride following the method of Lesser, R., and Gad, G., *Ber.*, **56**, 974(1923).

TABLE II.—PHYSICAL AND ANALYTICAL DATA OF SUBSTITUTED PHENOXYETHOXYETHYL CHLORIDES



R_1	Formula	Yield, %	B. P. (Uncorr.), ° C.	Chlorine, % Calcd.	Chlorine, % Found
4-(2-Methylbutyl-2)-2-chloro	$C_{15}H_{23}Cl_2O_2$	63	182–187/4 mm.	23.27	23.21
3,5-Dimethyl-4-chloro	$C_{12}H_{15}Cl_2O_2$	52	158–161/2 mm.	26.99	26.78
3,5-Dimethyl	$C_{12}H_{17}ClO_2$	63	116–118/5 mm.	15.35	15.26
4-(2-Methylbutyl-2)	$C_{15}H_{24}ClO_2$	68	188–192/12 mm.	12.96	12.83
4-(2-Methyl-4,4-dimethylpentyl-2)-2-chloro	$C_{18}H_{28}Cl_2O_2$	66	185–190/1.2 mm.	20.46	20.70
2,4-Di(pentyl-3)-6-chloro	$C_{26}H_{37}Cl_2O_2$	59	176–178.5/1.2 mm.	18.93	19.11
4-(2-Methylbutyl-2)-2,6-dichloro	$C_{15}H_{21}Cl_3O_2$	62	173–176/0.2 mm.	31.26	31.22

TABLE III.—PHYSICAL AND ANALYTICAL DATA OF THE TERTIARY AMINES[a]



R_1	Formula	Yield, %	B. P., (Uncorr.), ° C.	Chlorine, % Calcd.	Chlorine, % Found	Nitrogen, % Calcd.	Nitrogen, % Found
4-(2-Methylbutyl-2)-2-chloro	$C_{17}H_{28}ClNO_2$	58	196–198/4.7 mm.	11.34	11.19	4.47	4.41
4-(2-Methyl-4,4-dimethyl-pentyl-2)-2-chloro	$C_{20}H_{34}ClNO_2$	68	175–177/3 mm.	10.00	9.88	3.94	3.85
2,4-Di(pentyl-3)-6-chloro	$C_{27}H_{48}ClNO_2$	58	155–157/0.23 mm.	9.26	9.17	3.65	3.61

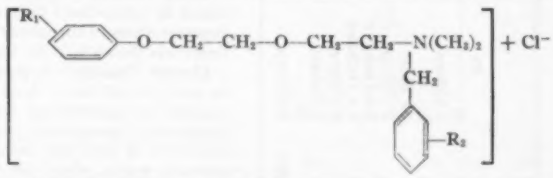
[a] The intermediates in which R = 3,5-dimethyl-4-chloro; 4-(2-methylbutyl-2)-2,6-dichloro; 4-(2-methylbutyl-2) were not isolated but used in a crude form for the preparation of the corresponding quaternaries.

All the quaternary ammonium salts described above were tested against *Staphylococcus aureus* (F. D. A.) and *Salmonella typhosa* (Hopkins strain) (Table V). The quaternary ammonium compound (IV) was tested against *Salmonella typhosa*, *Staphylococcus aureus*, *Salmonella paratyphi*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis* (bovine and human). The *Mycobacterium tuberculosis* organisms were the non-virulent, rapidly growing strains (ATCC. 607 and 599). These were grown in glycerol-peptone broth at 37° and incubated for three weeks (see Table VI, page 271).

Bacteriostatic and Fungistatic Activity.—Compound IV was tested for bacteriostatic and fungistatic activity against various organisms by the broth-dilution method as follows: a stock solution of the compound containing ten times the desired amount in the final dilution was prepared in sterile distilled water. Serial dilutions were made from the stock solution, and to 1.0 cc. of each dilution was added 9.0 cc. of media which had been previously seeded with a twenty-four hour culture of the test organism (0.1 cc./100 cc. broth). The series of tubes were then incubated at 37° for forty-eight hours, and on examination for visible growth, the critical concentration of the quaternary was observed. In the case of the fungi, saline suspensions of cultures were prepared from seeded plates and the broth inoculated (10 cc./300 cc. broth). The series of tubes were then incubated at 24–26° and aerated by means of a mechanical shaker for seventy-two hours.[1] The results are presented in Table VII (page 271).

[1] Initial experiments were carried out with the fungi using a three-week incubation period. It was observed that there was no change in the critical static concentrations between the seventy-two hour and the three-week incubation period. The seventy-two hour incubation period was then adopted for the remainder of our tests.

TABLE IV.—PHYSICAL AND ANALYTICAL DATA OF THE QUATERNARY AMMONIUM COMPOUNDS



R_1	R_2	Formula	Yield, %	M. P. (Uncorr.), ° C.	Nitrogen, % Calcd.	Nitrogen, % Found	Chloride Ion, % Calcd.	Chloride Ion, % Found
4-(2-Methylbutyl-2)-2-chloro	4-Chloro	$C_{24}H_{34}Cl_3NO_2$	78	105–108	2.96	2.81	7.50	7.62
3,5-Dimethyl-4-chloro	4-Chloro	$C_{22}H_{28}Cl_3NO_2$	76	78–80	3.24	3.31	8.21	8.31
3,5-Dimethyl	H	$C_{22}H_{29}ClNO_2$	74	125–128	3.86	3.73	9.64	9.71
4-(2-Methylbutyl-2)	H	$C_{24}H_{34}ClNO_2$	70	82–85	3.45	3.35	8.64	8.70
4-(2-Methyl-4,4-dimethylpentyl-2)-2-chloro	4-Chloro	$C_{27}H_{40}Cl_3NO_2$	72	128–131	2.71	2.67	6.87	6.75
2,4-Di(pentyl-3)-6-chloro	4-Chloro	$C_{29}H_{44}Cl_3NO_2$	54	[a]	2.57	2.43	6.43	6.31
4-(2-Methylbutyl-2)-2,6-dichloro	4-Chloro	$C_{24}H_{33}Cl_4NO_2$	51	[a]	2.75	2.68	6.97	6.81
4-(2-Methylbutyl-2)-2-chloro	2-Chloro	$C_{24}H_{34}Cl_3NO_2$	72	[b]	2.96	2.85	7.50	7.50
4-(2-Methylbutyl-2)-2-chloro	2,4-Dichloro	$C_{24}H_{33}Cl_4NO_2$	53		2.75	2.72	6.97	6.89
4-(2-Methylbutyl-2)-2-chloro	3,4-Dichloro	$C_{24}H_{33}Cl_4NO_2$	61	120–122	2.75	2.76	6.97	6.82

[a] Isolated as a gum.
[b] White crystalline hygroscopic solid.

TABLE V.—BACTERICIDAL ACTIVITY OF QUATERNARY AMMONIUM COMPOUNDS AGAINST *Staphylococcus aureus* AND *Salmonella typhosa*

No.	R_1	R_2	The Dilutions in Which Killing Occurs in Ten, but Not in Five Minutes—*Staphylococcus aureus*—Aqueous Solution		2% Peptone		*Salmonella typhosa*—Aqueous Solution		2% Peptone	
			20° C.	37° C.	20° C.	37° C.	20° C.	37° C.	20° C.	37° C.
I	3,5-Dimethyl	H	1–1,000	1–1,500	1–600	1–900	1–1,500	1–2,600	1–700	1–1,500
II	3,5-Dimethyl-4-chloro	4-Chloro	1–1,500	1–18,500	1–6,400	1–7,000	1–9,000	1–16,000	1–7,000	1–9,000
III	4-(2-Methylbutyl-2)	H	1–4,500	1–7,000	1–3,400	1–2,000	1–6,000	1–22,000	1–3,000	1–5,500
IV	4-(2-Methylbutyl-2)-2-chloro	4-Chloro	1–18,000	1–36,000	1–9,000	1–30,000	1–18,000	1–20,000	1–9,000	1–15,000
V	4-(2-Methylbutyl-2)-2-chloro	2-Chloro	1–12,000	1–23,500						
VI	4-(2-Methylbutyl-2)-2-chloro	2,4-Dichloro	1–28,000	1–21,000						
VII	4-(2-Methylbutyl-2)-2-chloro	3,4-Dichloro	1–28,000	1–59,000						
VIII	4-(2-Methylbutyl-2)-2,6-dichloro	4-Chloro	1–9,000	1–12,000	1–5,000	1–7,000	1–7,000	1–8,000	1–2,000	1–4,500
IX	2,4-Di(pentyl-3)-6-chloro	4-Chloro	1–15,000	1–14,000	1–6,000	1–10,000	1–14,000	1–7,000	1–1,000	1–1,200
X	4-(2-Methyl-4,4-dimethylpentyl-2)	H	1–23,000	1–44,000	1–15,000	1–30,000	1–23,000	1–43,000	1–3,000	1–5,000
XI	4-(2-Methyl-4,4-dimethylpentyl-2)-2-chloro	4-Chloro	1–34,000	1–42,000	1–6,500	1–18,000	1–9,000	1–51,000	1–1,500	1–2,500

RESULTS

The series reported here is not sufficiently complete to warrant the drawing of definite conclusions with regard to the influence of chloro- and alkyl-substitutions on the activity of quaternary ammonium compounds having two benzene nuclei. However, some effects are evident, and they tend to clarify the reasons for the conflicting statements in the literature.

Monochlorination of one or both rings is most effective in compounds having short side-chain substitutions in the phenoxy group, and much less effective or even decreases the antibacterial activity if the side chains are long. This is illustrated by the comparison of the activities of compounds I with II, III with IV, which show an increase, X with XI, and IV with IX, which do not show an increase but rather a decrease in activity as a result of ring chlorination. The introduction of a third chlorine atom in the 6-position of the phenoxy group, e.g., compound IV yielding compound VIII, results in a decrease of activity, while the introduction of the third chlorine atom in the benzyl group leads to an increase in activity and more so in the 4-position than in the 2-position (*cf.* compounds IV, VIII, VI, and VII). Comparison of V and VI suggests that activation is greater if the chloro substitution is in the 4- than if it is in the 2-position of the benzyl group. It seems that compounds having the 2- and 4-positions of the phenoxy groups occupied and having a chlorine in the 6-position have less activity than should be expected from the size of the substituting alkyl groups.

TOXICITY STUDIES

Acute Toxicity.—The LD_{50} for mice was determined by intravenous injection of 0.1 ml. of various concentrations of the quaternary compounds. The results are shown in Table VIII.

Chronic Toxicity.—Experiments were carried out on rats (50–100 Gm.); both sexes were equally represented in experimental and control groups. The experimental groups were supplied with a 0.1% solution of IV as their sole source of drinking water for nineteen weeks, while the control groups received tap water. The results are expressed as per cent gain in weight per group over their original weight (Fig. 1). At the end of nineteen weeks the animals were sacrificed and examination of their organs revealed no abnormality.

Irritation Studies. Since the type of compounds described are mostly used as surface antiseptics, it was of interest to compare a representative of this group with other quaternary antiseptics which are in use. The shaved back of a rabbit was divided into four areas. For each of the compounds tested, one area was used. Aqueous solutions containing 0.1 and 1% of each of the four compounds tested were painted on the skin of the back of the rabbits for five days and the rabbits were observed during this period and forty-eight hours later. Tests were also carried out according to the method described by Draize, Woodard, and Calvery (6) with the 1% solution. The results are given on Table IX.

Skin irritation tests using the same method on guinea pigs gave similar results.

TABLE VI.—BACTERICIDAL ACTIVITY OF 4-(2-METHYLBUTYL-2)-2-CHLOROPHENOXYETHOXYETHYL-4'-CHLOROBENZYLDIMETHYLAMMONIUM CHLORIDE AGAINST VARIOUS ORGANISMS

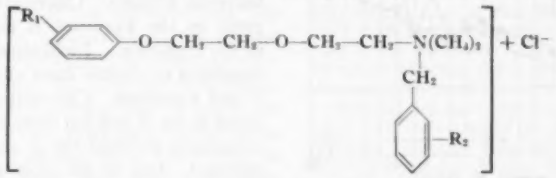
Organism	—The Dilutions in Which Killing Occurs in Ten but Not in Five Minutes— Aqueous Solutions 20° C.	Aqueous Solutions 37° C.	Peptone Solutions, 2% 20° C.	Peptone Solutions, 2% 37° C.
S. typhosa	1–18,000	1–20,000	1–9,000	1–15,600
S. aureus	1–18,000	1–36,000	1–9,000	1–30,000
S. paratyphi	1–20,000	1–36,000		
E. coli	1–8,000	1–18,000		
P. aeruginosa	1–2,000	1–12,500		
M. tuberculosis (bovine)	...	1–34,000		
M. tuberculosis (human)	...	1–22,000		

TABLE VII.—BACTERIOSTATIC AND FUNGISTATIC ACTIVITY OF 4-(2-METHYLBUTYL-2)-2-CHLOROPHENOXYETHOXYETHYL-4'-CHLOROBENZYLDIMETHYLAMMONIUM CHLORIDE AGAINST VARIOUS ORGANISMS

Test Organism	Broth	% Concn. of Compound Giving Growth	% Concn. of Compound Giving No Growth
Streptococcus pyogenes	Sabouraud's dextrose	0.000038	0.000066
Mycobacterium tuberculosis	Nutrient[a]	0.00065	0.0026
Pseudomonas aeruginosa	F. D. A.	0.0038	0.0067
Salmonella typhosa	Nutrient	0.0004	0.00056
Escherichia coli	Nutrient	0.0018	0.0024
Salmonella paratyphi	Nutrient	0.0017	0.0024
Staphylococcus aureus	Nutrient	0.00010	0.00014
Corynebacterium diphtheriae	Dextrose-protease[b]	0.000067	0.00013
Bacillus cereus	Nutrient	0.00012	0.0004
Trichophyton interdigitale	Sabouraud's dextrose	0.0006	0.0013
Aspergillis niger	Sabouraud's dextrose	0.0006	0.0013

[a] Plus 0.5% glycerin and 0.5% Tween 80.
[b] Plus 2% serum.

TABLE VIII.—ACUTE TOXICITY FOR MICE FOR QUATERNARY AMMONIUM COMPOUNDS



R_1	R_2	No. Mice	LD_{50} [Mg./Kg.]
4-(2-Methyl-4,4-dimethylpentyl-2)	H	18	29.5
4-(2-Methylbutyl-2)-2-chloro	4-Chloro	48	37.4
3,5-Dimethyl-4-chloro	4-Chloro	42	10.0
2-Isopropyl-5-methyl-4-chloro	4-Chloro	19	12.5
2,4-Di(pentyl-3)-6-chloro	4-Chloro	18	15.0
Alkyldimethylbenzylammonium chloride	...	24	18.3

TABLE IX.—SKIN IRRITATION TESTS ON RABBITS

No. Rabbit Sites	Test Type	Application	Concn., %	*A*	*B*	*C*	*D*
4	Paint	Twice daily for 5 days	0.1	±	++	− − −	− − −
8	Paint	Twice daily for 5 days	1	±	+++	++	+++
8	Paint	Twice daily for 5 days	1	±	+++	+	++
8	Occlusive dressing	Applied for 24 hours	1	±	++	− − −	− − −

− − − = not tested.
A = 4-(2-Methylbutyl-2)-2-chlorophenoxyethoxyethyl-4'-chlorobenzyldimethylammonium chloride.
B = Alkyldimethylbenzylammonium chloride.
C = Cetylpyridinium chloride.
D = 4-(2-Methyl-4,4-dimethylpentyl-2)-phenoxyethoxyethylbenzyldimethylammonium chloride.

TABLE X.—CONJUNCTIVAL IRRITATION TESTS

No. Animals	Application	Concn., %	*A*	*B*
Guinea Pigs				
3	5 drops twice daily for 1 wk.	0.05	Left eye, normal	Right eye, normal
Rabbits				
2	5 drops twice daily for 1 wk.	0.05	Normal	Normal
2	5 drops three times daily for 1 wk.	0.003	Slight irritation when applied—otherwise normal	Slight irritation when applied—otherwise normal

A = 4-(2-Methylbutyl-2)-2-chlorophenoxyethoxyethyl-4′-chlorobenzyldimethylammonium chloride.
B = Alkyldimethylbenzylammonium chloride.

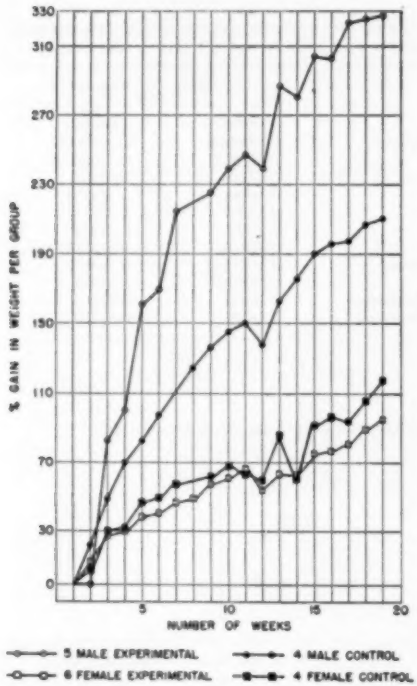


Fig. 1.—Chronic toxicity of 4-(2-methylbutyl-2)-2-chlorophenoxyethoxyethyl-4′-chlorobenzyldimethylammonium chloride.

Patch tests on 11 humans using 1% aqueous solutions showed that slight irritation is caused in most individuals by all compounds tested. The percentage of cases showing irritation was as follows: with alkyldimethylbenzylammonium chloride 64%; with 4-(2-methylbutyl-2)-2-chlorophenoxyethoxyethyl-4′-chlorobenzyldimethylammonium chloride 27%; with 4-(2-methyl-4,4-dimethylpentyl-2)-phenoxyethoxyethylbenzyldimethylammonium chloride 27%.

Conjunctival irritation tests were carried out on rabbits and guinea pigs. The results given in Table X indicate that there was no significant irritation at the concentrations tested with either of the compounds studied.

SUMMARY

A series of new quaternary compounds of the aryloxyethoxyethylbenzyldimethylammonium chloride type has been synthesized and the effect of simultaneous alkylation of the phenoxy group and chlorination of one or both benzene rings on the antibacterial activity has been determined. The enhancing effect of chlorination is relatively greater in compounds having short alkyl substituents in the phenoxy group. If the alkyl substitutions in the phenoxy group are greater than that corresponding to eight carbon atoms, chlorination decreases rather than enhances the antibacterial activity. Chlorination of the benzyl group in the 4-position is more effective than in the 2-position. Dichlorination in the 3- and 4-position is slightly more effective than in the 2- and 4-position. Chlorination of the phenoxy group in the 6-position decreased the activity of compounds in which the 2- and 4-positions were occupied. One of the most active compounds, 4-(2-methylbutyl-2)-2-chlorophenoxyethoxyethyl-4′-chlorobenzyldimethylammonium chloride, was extensively studied. The spectrum of its antimicrobial action was determined and its toxicity and results of irritation tests were reported.

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Comparative Pharmacology of Arterenol, Epinephrine and Isopropylarterenol*

By GRAHAM CHEN, RUTH PORTMAN, DAVID RUSSELL, and CHARLES R. ENSOR

A comparison has been made of the cardiovascular, bronchodilatory, spasmogenic, hyperglycemic, and adrenal-stimulating effects of arterenol, epinephrine, and isopropylarterenol in equivalent dose levels. Epinephrine is twice as potent as arterenol in producing vasoconstriction of the vascular bed of skeletal muscles. Epinephrine acts like arterenol to produce the vasopressor, and like isopropylarterenol to produce the vasodepressor effect. Isopropylarterenol is three times more effective than epinephrine in prevention of histamine-induced bronchospasm in guinea pigs, while arterenol is $^1/_{60}$ as effective as epinephrine. Arterenol, like epinephrine, causes contraction of isolated guinea pig's seminal vesicle, but is less active. Isopropylarterenol is devoid of a spasmogenic action. The hyperglycemic activity of arterenol is greater than that of isopropylarterenol, but less than that of epinephrine. They are equally influential on the ascorbic acid metabolism of the adrenals.

ARTERENOL, epinephrine, and isopropylarterenol have for some time attracted the attention and interest of investigators in the field of pharmacodynamics and therapeutics, especially from the relationship of chemical constitution and pharmacological action(1,2). Arterenol is primarily a sympathomimetic excitatory, while isopropylaterenol is a sympathomimetic inhibitory agent. Epinephrine possesses an inhibitory action greater than arterenol but less than isopropylarterenol. That arterenol is present in small amounts in the adrenal medulla and that it is probably liberated from the sympathetic nerves upon stimulation (3) have given rise to speculations as to what role arterenol may play in the synthesis of epinephrine in the adrenal gland and in the transmission of adrenergic excitatory nerve impulses (4). By their similar pharmacological actions to epinephrine, arterenol and isopropylarterenol have been suggested for therapeutic uses in conditions where epinephrine is indicated. Both from the pharmacodynamic and the therapeutic standpoint, therefore, it is important to know the differences in their pharmacological activities. This led us to make a quantitative comparison of the sympathomimetic properties of arterenol, epinephrine, and isopropylarterenol including their cardiovascular, bronchial dilatory, spasmogenic, hyperglycemic, and adrenal cortico-stimulating actions. Some of the experimental procedures employed are different from those hitherto given in the literature. The results are presented in this report.

EXPERIMENTAL

l-Epinephrine,[1] *l*-arterenol bitartrate, and *dl*-isopropylarterenol hydrochloride were used. In the results, the doses or concentrations are given in terms of the base.

Methods and Results

Vasoconstriction.—Sprague-Dawley rats weighing between 100 and 150 Gm. were used. Local vasoconstriction was determined by the delay in onset of convulsions of rats given intramuscularly a lethal dose of strychnine nitrate (10 mg./Kg.) together with the test compound. The time of survival was also recorded.

A comparison of the vasoconstricting effects of *l*-epinephrine and *l*-arterenol is presented graphically in Fig. 1, in which the mean time of delay in onset of convulsions or the mean increase in survival time is plotted against the concentration of epinephrine and arterenol. It is seen that in equivalent dose levels, epinephrine vasoconstriction is much greater than that of arterenol. This is in agreement with the results of others obtained from vasoconstriction in perfused rabbit ear and from the enhanced effect of intradermal procaine anesthesia in guinea pigs (5). From the linear portion of the two curves for epinephrine and arterenol, their relative vasoconstricting effects at equal dosages have been calculated. This is obtained by a statistical treatment of data, ten determinations for each concentration, with a factorial design and analysis of variance (6, 7). Arterenol was thus found to be approximately one-half as effective as epinephrine in vasoconstricting activity —0.537 ± 0.052 (S. E.) calculated from the time of delay in onset of convulsions, 0.493 ± 0.068 from the increased time of survival.

Isopropylartenenol is a vasodilating agent; thus it produces an increase in the rate of absorption of strychnine into the circulation when they are injected together intramuscularly. The mean survival time of 10 rats receiving 5 mg./Kg. of strychnine nitrate is fourteen minutes and six seconds; it is three minutes and ten seconds when the same amount of strychnine is injected with 1:100,000 isopropylarterenol.

Blood Pressure.—Dogs or cats under pentobarbital anesthesia were used. Blood pressure was measured from the carotid artery; injections were given in the femoral vein. In accord with the observation of others, *l*-arterenol produces a greater hypertension than does *l*-epinephrine. Cocaine,

* Received October 12, 1950, from the Research Department of Parke, Davis and Company, Detroit, Mich.

[1] *l*-Epinephrine was made available to us by Mr. F. H. Tendick of Parke, Davis and Co. It contains less than 0.10% of arterenol.

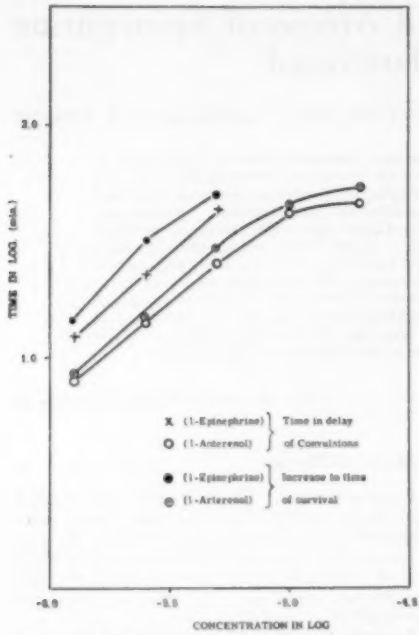

Fig. 1.—The vasoconstricting effect of epinephrine and arterenol is shown by the delay in onset of convulsions and in time of death following intramuscular injection in rats of 10 mg./Kg. of strychnine nitrate with various concentrations of arterenol and epinephrine.

diphenhydramine and a number of other substances potentiate the hypertensive response to epinephrine and arterenol (8–11). Since the vasoconstriction induced by epinephrine in the muscles and the skin (5) is greater than that by arterenol, the difference in hypertensive response to the two agents must be due to a different action on some other sites of the cardiovascular system, such as the heart. The observations on their relative inotropic and chronotropic effects on the heart by different investigators are, however, not in complete agreement (5, 12–15).

The visceral vessels are affected by epinephrine and arterenol differently in different animals, depending upon the nature of their vascular supply. In most dogs, a marked vasodilatation of the viscera is induced by epinephrine, thus lowering the rise of blood pressure due to its vasopressor action. This is shown in Fig. 2 by the greater hypertensive effect of epinephrine after evisceration. The absence of viscera did not result in a difference in hypertension by arterenol. The vasodepressor effect of epinephrine on other organs is seen after adrenergic blockade with N-(2-bromoethyl)-N-ethyl-1-naphthalene methylamine HBr (SY-28). In some cats, the vasopressor effect of epinephrine is changed into a vasodepressor effect following evisceration, while the rise of blood pressure to arterenol is only slightly decreased (Fig. 3). Apparently, vasoconstriction is the predominant visceral response to epinephrine in these animals.

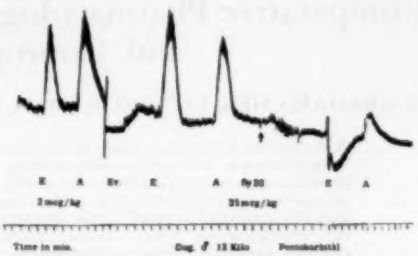

Fig. 2.—There was a greater epinephrine hypertension but no difference in arterenol hypertension following evisceration. The vasopressor response to epinephrine was reversed, that to arterenol suppressed, after adrenergic blockade with SY-28. *E* = Epinephrine, *A* = Arterenol, *Ev* = Evisceration.

Whether or not arterenol possesses a vasodilating action has been questioned. Only a slight vasodepressor response to arterenol is elicited under adrenergic blockade. The vasodepressor component of epinephrine and the depressor response to isopropylarterenol may be converted to a pressor response by ergotamine, diphenhydramine, and by pilocarpine and other cholinergic agents (10).

Bronchial Dilatation.—The bronchial-dilating activities of arterenol, epinephrine, and isopropylarterenol were appraised by the prevention of histamine-induced fatal bronchospasm in guinea pigs (16, 17). The test compound was given, together with histamine in the aerosol. Four doses of each compound, 20 animals per dose, were used to obtain the quantity that will prevent death in 50% of the animals breathing a fatal concentration of histamine. The PD_{50}'s given in Table I were interpolated from the probit-log dose regression lines. The data indicate that isopropylarterenol is three times more effective than epinephrine in dilating histamine-constricted bronchioles. The bronchial-dilating activity of arterenol is about $^1/_{60}$ that of epinephrine. It is of interest to note that epinephrine and isopropylarterenol are equally effective clinically in relieving some types of bronchial asthma (18).

Spasmogenic Effect.—Isolated seminal vesicle of guinea pig in oxygenated Ringer-Locke solution at 37° was used. Epinephrine and arterenol or

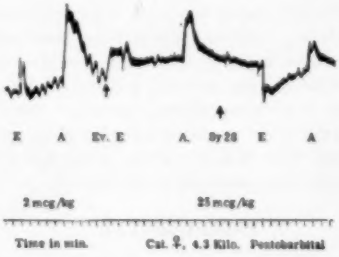

Fig. 3.—The vasopressor effect of epinephrine was changed into a vasodepressor effect following evisceration, while the vasopressor effect of arterenol was slightly decreased. SY-28 reversed the epinephrine pressor to a depressor response and reduced the pressor effect of arterenol.

TABLE I.—BRONCHODILATING EFFECT OF ARTERENOL, EPINEPHRINE, AND ISOPROPYLARTERENOL AS DETERMINED BY PREVENTION OF HISTAMINE-INDUCED FATAL BRONCHOSPASM IN GUINEA PIGS

Compound	Arterenol	Epinephrine	Isopropylarterenol
PD_{50}, μg./L.	2,750	45.3	15
Relative activity	1/60	1	3

isopropylarterenol were added to the bath alternately. The tissue was allowed to rest for ten minutes between each determination. As the data in Table II show, epinephrine produces a greater contraction than does arterenol. Isopropylarterenol is ineffective. The response of seminal vesicle is thus similar to that of other smooth musculatures (5, 19) except the pregnant uterus of the cat (20). The latter is said to respond more actively to arterenol than to epinephrine.

Hyperglycemic Action.—Rabbits without food for sixteen hours were used. The hyperglycemic agent was given by subcutaneous, intravenous, or intracisternal administration. Intracisternal injection was made without anesthesia with a 27-gauge needle inserted into the fourth ventricle at the back of the neck between the atlas and the base of the occipital bone. This can easily be performed by having the animal in an upright position, its head downward, and placing the needle cephalically and obliquely toward the ventricle. Before the solution was injected, a small amount of clear cerebrospinal fluid was withdrawn into the syringe to be sure that it was not in any way given directly into the blood stream.

Blood glucose determinations were carried out by the method of Miller and van Slyke (21). Blood samples in duplicates were drawn one-half, one, two, and three hours after administration of the hyperglycemic agent. As a rule, a maximal hyperglycemic response is reached between two and three hours after subcutaneous or intracisternal injection and between one-half and one hour after intravenous injection.

Values of the increase in blood glucose of rabbits receiving arterenol, epinephrine, or isopropylarterenol are recorded in Table III. The data indicate that epinephrine is the most active, while isopro-

TABLE II.—SPASMOGENIC EFFECT OF ARTERENOL, EPINEPHRINE, AND ISOPROPYLARTERENOL ON ISOLATED GUINEA PIG'S SEMINAL VESICLES

	Bath Concentration, μg./Cc.											
	l-Epinephrine					*l*-Arterenol					*dl*-Isopropyl Arterenol		
Experiment	0.5	1	2	4	10	0.5	1	2	4	10	1	10	20
	Contractions, Mm.[a]												
1	17	38	48	49	..	11	24	41	43	..	0	0	0
2	22	36	41	..	..	16	30	36	..	..	..	..	..
3	18	36	28	32	..	3	13	18	24	..	..	..	..
4	18	26	23	32	31	9	15	20	24	31	0	0	0
5	21	59	81	82	82	9	58	70	72	72	0	0	0
Mean	19.2	43	44	49	56	9.6	28	37	41	52	0	0	0

[a] Mm. as recorded on the drum.

TABLE III.—THE HYPERGLYCEMIC EFFECT OF EPINEPHRINE, ARTERENOL, AND ISOPROPYLARTERENOL BY SUBCUTANEOUS, INTRAVENOUS, AND INTRACISTERNAL INJECTION

Dose, Mg./Kg.	Glucose, Mg. %, Mean ± S. E.	Dose, Mg./Kg.	Glucose, Mg. %, Mean ± S. E.	Dose, Mg./Kg.	Glucose, Mg. %, Mean ± S. E.
	A. Subcutaneous Injection[a]				
0.05	44.3 ± 6.5 (8)[b]	0.25	40.0 ± 2.9 (4)	0.40	37.7 ± 1.5 (4)
0.10	76.0 ± 5.6 (5)	0.50	56.8 ± 11.1 (4)	4.00	65.8 ± 12.1 (4)
0.20	174.4 ± 16.3 (5)	1.25	184.8 ± 45.5 (5)	16.00	74.0 ± 7.7 (4)
1.00	259.0 ± 9.4 (6)	2.50[c]	182.8 ± 3.1 (4)	32.00[c]	168.0 ± 27.8 (4)
$K = 260$,[d] $a = 7.1$, $b = -10$		$a = 6.4$, $b = -1.1$		$a = 6.0$, $b = -0.75$	
	B. Intravenous Injection,[a] 0.1 Cc./Kg.				
0.025	28.5 ± 4.8 (6)	0.025	11.1 ± 2.6 (6)	0.20	25.5 ± 3.6 (6)
0.05	33.0 ± 6.6 (6)	0.05	16.5 ± 3.5 (6)	0.40	40.8 ± 7.8 (6)
0.10[c]	54.1 ± 8.1 (7)	0.10[c]	14.5 ± 1.6 (6)	0.80[c]	50.8 ± 9.5 (6)
	C. Intracisternal Injection,[a] 0.1 Cc./Kg.				
0.10	97.0 ± 26.8 (6)	0.10	35.8 ± 8.5 (6)	0.10	41.8 ± 7.5 (6)
0.20	118.3 ± 31.5 (6)	0.20	53.2 ± 9.0 (6)	0.20	31.6 ± 4.5 (6)
0.40	148.2 ± 25.4 (6)	0.40	54.0 ± 6.4 (6)	0.40	31.5 ± 7.4 (6)
0.80[c]	201.2 ± 34.5 (6)	0.80[c]	64.5 ± 9.3 (6)	0.80[c]	44.1 ± 11.4 (6)
$K = 260$,[d] $a = 2.6$, $b = -3.1$					

[a] 0.9% Salt control (0.1 cc./Kg.): mean = 19.2 ± 4.9 (6).
[b] Number of rabbits used.
[c] Maximal tolerated dose.
[d] $Y = K/(1 + ax^{bx})$. Y = Increase of blood glucose; x = dose of hyperglycemic agent; a, b, K = constants.

TABLE IV.—ASCORBIC ACID CONTENTS OF ADRENALS OF RATS RECEIVING EPINEPHRINE, ARTERENOL, AND ISOPROPYLARTERENOL[a]

Dose (Subcutaneous), Mg./Kg.	Epinephrine		Arterenol		Isopropylarterenol	
	Mg./100 Gm.	Difference	Mg./100 Gm.	Difference	Mg./100 Gm.	Difference
0 (Control)	441	...	387	...	440	...
0.01	380	13.8	310	19.9	351	20.2
0.05	366	17.0	291	22.2	334	24.1
0.10	342	22.4	321	26.6	246	44.1
0.50	288	34.7	220	43.1	222	49.5
0 (Control)	333	...	333	...	333	...
1.0	229	31.2	191	42.6	179	46.2

[a] All values are averages of four determinations of both adrenal glands.

pylarterenol is the least active hyperglycemic agent. The hyperglycemic activity of arterenol is approximately 0.17 and that of isopropylarterenol is 0.006 the activity of epinephrine by subcutaneous injection. The values are in agreement with those recently reported (23, 24). The epinephrine hyperglycemia by intracisternal injection is less than that by subcutaneous injection.

Except for the difference in potency, the dose-response relationship is the same for the three compounds. This is shown by fitting the data to the logistic function:

$$Y = \frac{K}{1 + ae^{bx}}$$

Y = hyperglycemia
x = dose
K = maximal response
a, b = constants

A maximal glycemic response could be obtained only by a nonlethal subcutaneous dose of epinephrine. With this value of K, the constants a and b were estimated graphically (22) for epinephrine, arterenol, and isopropylarterenol by subcutaneous injection, and for epinephrine by intracisternal injection (Table III). Thus, it appears that the same mechanism is involved in producing hyperglycemia by the three agents.

Effect on Adrenal Cortex.—The effect of epinephrine, arterenol, and isopropylarterenol on the ascorbic acid metabolism of the adrenals was investigated. For this, male Sprague-Dawley rats weighing between 150 and 200 Gm. were used. Ascorbic acid content was determined by titration with 2,6-dichlorophenolindophenol (25). The test compound was injected subcutaneously. Two hours later, as soon as the rat was put under pentobarbital anesthesia, the adrenal glands were dissected, immediately weighed, and extracted with 2 cc. of 3% HPO_3, in a mortar. The content and washings, three times with 1 cc. of 3% HPO_3, were transferred quantitatively into a 15-cc. centrifuge tube. After centrifugalization, the entire supernatant fluid was poured into a test tube and titrated with the dye for ascorbic acid.

The results given in Table IV are in mg. of ascorbic acid per 100 Gm. of fresh tissue. The differences in ascorbic acid contents between controls and the adrenals of treated animals are expressed in percentages. In so far as the site and the mechanism of action of these agents on the metabolism of ascorbic acid are not clear (26, 27), it suffices to state that epinephrine, arterenol, and isopropylarterenol are equally influential on the metabolism of the adrenals.

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Acute and Chronic Toxicity of Potassium Acid Saccharate*

By ANTHONY M. AMBROSE†,‡

The acute oral or intraperitoneal toxicity of potassium acid saccharate is extremely low. No toxic effects or fatalities were observed in rats receiving as much as 2 Gm./Kg. in a single dose of a solution of potassium acid saccharate previously neutralized to reduce irritation. Rats fed diets containing 2 per cent or less potassium acid saccharate for approximately two hundred and ten days showed no untoward effect as judged by general appearance, growth, the amount of food consumed, mortality, organ weights, and microscopical examination of visceral organs.

THE similarity in physical and chemical properties of potassium acid saccharate to potassium acid tartrate and citric acid has suggested the possible use of the former as replacement for the latter two acidifying agents. Although not a complete substitute for either of these agents, potassium acid saccharate is of considerable interest as an acidifier for baking powder, in the fruit acid industry, and in medicine. In view of the critical shortage of tartaric and citric acids, a wider application of potassium acid saccharate is anticipated, particularly in the formulation of dietary preparations. Obviously, before potassium acid saccharate is used in place of tartrates and citrates in foods or in medicine, detailed toxicological studies should be available. To our knowledge no such studies have been made other than those reported by Carr (1), in which rats were permitted to ingest food containing 5 per cent potassium acid saccharate for periods up to seven months without any appreciable effects on growth, conception, or pregnancy, and without significant incidence of pathological lesions in the tissues studied. In the present studies, data have been obtained on the acute toxicity by single oral or intraperitoneal administration of large doses, and on the effects of small amounts administered as an integral part of the diet for approximately two hundred and ten days.

Saccharic acid is a hydroxy dibasic acid readily obtained by the oxidation of glucose (2). The potassium salt used was prepared and supplied by the Northern Regional Research Laboratory of this Bureau.

* Received November 7, 1950, from the Pharmacology Laboratory, Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Dept. of Agriculture, Albany 6, Calif.

† With the technical assistance of Elton K. Doxtader and Paul V. Marsh.

‡ The histopathological examination and evaluation of the tissues of rats reported herein were made by Dr. Alvin J. Cox, Jr., of the Department of Pathology, Stanford University Medical School, San Francisco, Calif.

EXPERIMENTAL PROCEDURES AND RESULTS

Acute Toxicity.—The acute toxicity of potassium acid saccharate, after single administration, was determined in albino rats weighing 115–150 Gm. each. The rats received the test dose either orally (by stomach tube) or intraperitoneally. In order to reduce gastric or intraperitoneal irritation, solutions of potassium acid saccharate were neutralized with sodium hydroxide to pH 7 just before administration. The dosages administered were calculated in terms of saccharic acid.

Three groups of 10 rats each were given, by stomach tube, 0.5, 1, and 2 Gm./Kg. (equivalent to 0.59, 1.18, and 2.36 Gm. potassium acid saccharate), and a similar number of rats were given the same dosage intraperitoneally. The rats were observed frequently during the first four hours following administration, and daily for fourteen days. No toxic reactions were observed immediately or during fourteen days after administration by either route.

Higher dosages by oral administration were not attempted, since it is quite unlikely that any one would ingest dosages of potassium acid saccharate in excess of 2 Gm./Kg.

Chronic Toxicity.—The cumulative effects, if any, of potassium acid saccharate in rats might be reflected in growth, changes in body weight, or in histological changes of visceral organs. Studies on continued oral ingestion by voluntary eating of food containing potassium acid saccharate were conducted with weanling male albino rats from our inbred colony. Rats weighing 40–45 Gm. each were placed on diets containing varying concentrations of saccharate for approximately two hundred and ten days. Eight groups of five each were used. Each group was placed in a rectangular cage containing wood shavings as bedding.

The respective diets were placed in special feeding devices, which reduced spilling and wastage to a minimum. The feeding device consisted of one-pint, wide-mouth Mason-type jars, in which was inserted a piece of $^1/_4$-in. wire mesh screening, which floated on top of the food. The jars were fitted to zinc screw-caps having a 2-in. hole to which one-half of a 90° 2-in. galvanized conduit pipe elbow was soldered. The other end of the conduit pipe was passed through the side of the cage. The whole feeding assembly was securely attached to the outside of the cage.

TABLE I.—SUMMARY ON THE CHRONIC TOXICITY OF POTASSIUM ACID SACCHARATE (KHS) AND POTASSIUM ACID TARTRATE (KHT) IN MALE RATS. TIME: APPROXIMATELY 210 DAYS

Supplement	No. of Rats—At Start	No. of Rats—At End	Average Weight, Gm.—At Start	Average Weight, Gm.—At End	Average Weight, Gm.—Increase ± S. E.[a]	Food Consumption, Gm./Rat/Day
Control	5	5	43.8	277.8	234.0 ± 16.36	13.9
0.01% KHS	5	5	42.4	288.2	245.8 ± 12.13	13.3
0.05% KHS	5	5	41.2	298.8	257.6 ± 9.97	13.1
0.1% KHS	5	5	41.4	285.4	244.0 ± 10.27	13.7
0.5% KHS	5	5	41.0	292.2	251.2 ± 7.70	14.4
1% KHS	5	5	41.8	303.8	262.0 ± 8.33	14.7
2% KHS	5	5	42.2	301.4	259.2 ± 9.45	14.2
1.52% KHT	5	5	41.8	287.0	245.2 ± 9.39	13.6

[a] S. E. = standard error of the mean.

Ground commercial dog food (Purina) served as the control as well as the basic diet to which potassium acid saccharate or potassium acid tartrate was added. To supply comparative data on ingestion of potassium, one group was fed the basic diet containing 1.52% of potassium acid tartrate. The potassium content of this diet and the one containing 2% potassium acid saccharate were equal. The general design of the experiment is presented in Table I. Free access to food and water was permitted at all times. Weights of individual rats in each group and their food consumption were determined weekly. Frequent observations were made on the general well-being of all rats and on consistency of fecal droppings.

Growth curves for the control rats and each group of rats on the various diets are presented in Fig. 1. Table I summarizes data on average initial and final body weights for each group of rats and average daily food consumption per rat. Table II summarizes data on average organ weights. The *t*-test was used to compare mean values of the control group with the mean values of groups of rats on the diets containing varying concentrations of supplements.

Throughout the period there was no evidence that any of the diets was toxic, as determined by gross appearance, food consumption, growth (Fig. 1), or survival. As a matter of fact, rats on the supplements made slightly greater gains in body weight than did the control rats (Fig. 1). However, this slight difference in growth was not statistically significant (Table I).

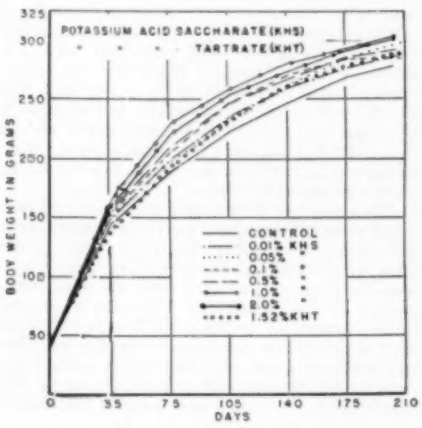

Fig. 1.—Growth curves.

Differences in average daily food consumption for control rats and of those on the various supplements (Table I) were not statistically significant. The average daily intake of potassium acid saccharate for rats on the 1 and 2% diets was approximately 425 and 850 mg./Kg., respectively. Diets containing 0.05, 0.1, and 0.5% potassium acid saccharate resulted in some evidence of softening of the stools (laxative), but this action was not as demonstrable as it was in rats on the diets containing 1 and 2% potassium acid saccharate or 1.52% potassium acid tartrate. The mild laxative effect consisted of only a slight softening of the stools. In none of the rats was there any evidence of softening of the stools to the point of staining the fur.

On completion of the feeding experiment there was no apparent difference in the general appearance between control rats and those that received the supplements, as could be determined subjectively. At autopsy, no gross post-mortem changes were discernible in any of the rats that could be ascribed to the supplements fed. In a few of the rats there was some evidence of hemorrhages of the lungs. The incidence of lung hemorrhage was no greater in rats on experimental diets than in rats on basic diet alone.

Paraffin sections of the following tissues stained with hematoxylin-eosin were studied microscopically—thyroid, heart, lungs, stomach, small intestine, adrenal, liver, spleen, pancreas, kidneys, urinary bladder, and testes. In these structures, no difference could be seen between rats on dietary supplements and the controls, except for the following: hemosiderin was found in all of the spleens. The lungs of rats which appeared hemorrhagic at necropsy showed focal intra-alveolar hemorrhage on microscopical examination of stained sections. A majority of the rats showed varying degrees of polymorphonuclear leucocytic infiltration in the submucosa of the stomach and esophagus near the junction with the stomach. This did not parallel the intake of supplements fed. It is probable that none of the changes described had any relation to ingestion of diets containing potassium acid saccharate or tartrate.

Organ weights were made routinely on all rats. The average weights of liver, spleen, kidneys, adre-

TABLE II.—STATISTICAL TREATMENT OF DATA ON ORGAN WEIGHTS OF MALE RATS ON DIETS CONTAINING POTASSIUM ACID SACCHARATE (KHS) AND POTASSIUM ACID TARTRATE (KHT). DATA FROM FIVE RATS IN EACH GROUP

Supplement	Liver, Gm.	Spleen, Gm.	Kidneys, Gm.	Adrenals, Gm.	Heart, Gm.	Testes, Gm.
	Mean Wt. ± S. E.[a]	Mean Wt. ± S. E.	Mean Wt. ± S. E.	Mean Wt. ± S. E.	Mean Wt. ± S. E.	Mean Wt. ± S. E.
Control	9.44 ± 0.876	0.459 ± 0.048	1.956 ± 0.092	0.031 ± 0.0017	0.935 ± 0.041	2.890 ± 0.139
0.01% KHS	9.57 ± 0.444	0.487 ± 0.014	2.061 ± 0.082	0.029 ± 0.0025	0.984 ± 0.031	2.984 ± 0.092
0.05% KHS	10.43 ± 0.613	0.520 ± 0.030	2.025 ± 0.309	0.030 ± 0.0021	0.998 ± 0.035	3.070 ± 0.106
0.1% KHS	9.43 ± 0.336	0.420 ± 0.019	2.023 ± 0.097	0.027 ± 0.0009	0.972 ± 0.053	3.228 ± 0.061
0.5% KHS	10.51 ± 0.158	0.447 ± 0.017	2.266 ± 0.077[b]	0.028 ± 0.0009	0.965 ± 0.031	3.260 ± 0.050[b]
1.0% KHS	11.62 ± 0.374[b]	0.452 ± 0.042	2.236 ± 0.063[b]	0.027 ± 0.0008	0.973 ± 0.028	3.190 ± 0.055
2.0% KHS	9.70 ± 0.254	0.461 ± 0.010	2.270 ± 0.054[b]	0.030 ± 0.0006	1.006 ± 0.031	3.398 ± 0.068[b]
1.52% KHT	11.04 ± 0.406[b]	0.431 ± 0.035	2.048 ± 0.060	0.033 ± 0.0014	0.960 ± 0.017	2.920 ± 0.086

[a] S. E. = standard error of the mean.
[b] Significant, P = 0.05.

nals, heart, and testes are summarized in Table II. It is evident that the values for spleen, adrenals, and heart compare favorably with those of the control group, whereas the average values for the other organs do not. When the organ weights are calculated on a gram-per-100-Gm. body-weight basis, the differences in values for liver, kidney, and testes are not so striking as those presented in Table II. However, a significant difference exists between the livers of control rats and those on 1% potassium acid saccharate, and on 1.52% potassium acid tartrate. The same is true for the kidneys of rats on the three highest doses of potassium acid saccharate and the testes of rats on 0.1, 0.5, and 2% potassium acid saccharate. It is doubtful if the slight differences in weights of the liver, kidney, and testes are of any particular significance in view of the negative microscopical findings in these organs.

SUMMARY AND CONCLUSIONS

1. A study of the acute and chronic toxicity of potassium acid saccharate has been made in rats.

2. The acute oral or intraperitoneal toxicity of potassium acid saccharate is extremely low. No toxic effects or fatalities were observed in rats receiving as much as 2 Gm./Kg. in a single dose of a solution of potassium acid saccharate previously neutralized to reduce irritation.

3. Rats fed diets containing 2 per cent or less potassium acid saccharate for approximately two hundred and ten days showed no untoward effect as judged by general appearance, growth, the amount of food consumed, mortality, organ weights, and microscopical examination of visceral organs.

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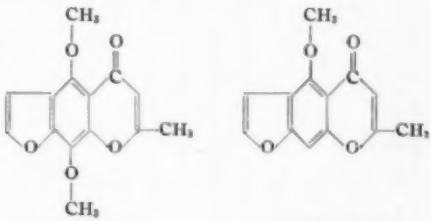
Khellin. Part I. Ultraviolet, Infrared, and Polarographic Studies on Three Principles Isolated from *Ammi visnaga* Lam.*,†

By S. D. BAILEY, P. A. GEARY, and A. E. deWALD‡,§

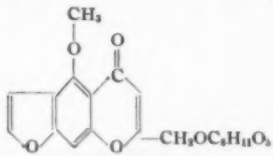
The ultraviolet and infrared absorption, as well as the polarographic half-wave potentials, are reported for khellin, visnagin, and khellol-glucoside; three principles found in the seeds of the plant *Ammi visnaga* Lam. These properties have been found useful in the identification and assay for the principles in plant extracts and pharmaceutical preparations. Ultraviolet absorption in 95 per cent ethanol and cyclohexane have been reported for each of the principles in the region from 210 to 380 mμ. Khellin, visnagin, and khellol-glucoside may be reduced at the dropping mercury electrode and the polarograms obtained have been found useful in assay procedures. Infrared absorption curves of khellin, visnagin in chloroform, and khellol-glucoside as a Nujol mull, have been obtained. Comparison of the infrared spectra of synthetic and natural khellin and visnagin provides further confirmation to the structures assigned to these compounds.

PREVIOUS investigators have reported on the isolation, pharmacological action, and syntheses (1–18) of khellin as well as other principles isolated from the seeds of *Ammi visnaga* Lam. The literature provides little, however, on characterizing physical and chemical properties, with the exception of a colorimetric identification test (19), and the ultraviolet spectrophotometric studies of Samaan, *et al.* (20, 21). Due to the similarity of structure of khellin, visnagin, and khellol-glucoside, more specific characterizing properties were investigated, since extracts from the plant may contain these three principles. The present paper gives several properties which have not been previously reported and which have proved useful in identification and assay tests. These properties include ultraviolet and infrared absorption spectra, as well as polarographic half-wave potentials. Part II deals with the use of these properties in analytical determinations of plant fractions and pharmaceutical preparations.



Khellin
(I)

Visnagin
(II)



Khellol-Glucoside
(III)

The accepted structures for khellin (I), visnagin (II), and khellol-glucoside (III) are shown in the opposite column.

Khellin was found by Späth and Gruber (16) to be 2-methyl-5,8-dimethoxyfuranochromone, while visnagin is 2-methyl-5-methoxyfuranochromone, differing from khellin only in the absence of a methoxy group in the eighth position. The glucoside of visnagin, commonly referred to as khellol-glucoside, has the methyl in the 2-position connected to the glucose moiety. The recent syntheses of khellin from dimethoxy resorcinol by Clarke and Robertson (13), and from khellinone by Geissman (14), confirm the structures reported by Späth and Gruber. Visnagin has been completely synthesized by Gruber (15) and has been obtained in these laboratories from khellol-glucoside by a process of degradation and synthesis.[1] The present study lends further support to the assigned structures of khellin and visnagin by a comparison of the infrared spectra of natural and synthetic materials.

* Received March 20, 1951, from the Research Division of Smith, Kline and French Laboratories, Philadelphia, Pa.
† Presented to the Pharmacy Subsection of the American Association for the Advancement of Science, Cleveland meeting, December 28, 1950.
‡ Research Division, Smith, Kline and French Laboratories, Philadelphia, Pa.
§ The authors are indebted to Dr. T. A. Geissman, University of California, Los Angeles, for the sample of synthetic khellin and to Dr. H. L. Dinsmore of Smith, Kline and French Laboratories for running the infrared curves.

[1] Unpublished work of Dr. P. N. Craig of Smith, Kline and French Laboratories.

EXPERIMENTAL

Both plant and synthetic khellin were purified by repeated crystallizations from water and found to have a melting point of 154.0 to 154.5°. A rapid crystallization of khellin from a water-ethanol mixture gave crystalline structures as shown in the microphotograph of Fig. 1, *A;* while a slower crystallization produced long, needle-like crystals as shown in Fig. 1, *B*. Visnagin, plant and synthetic, recrystallized from petroleum ether (77–177° b. p.), had a melting point of 140.5 to 141.5°. Recrystallization of visnagin from water-ethanol gave a bulky, fibrous mass as shown in Fig. 1, *C;* however, when crystallized from benzene-petroleum ether, larger pyramidal crystals as in Fig. 1, *D* were formed. Samaan (7) in 1930 reported that khellin (visammin[1]) crystallizes from hot concentrated aqueous solution on cooling as long, white, cotton-like hairs. It is our observation that this is more descriptive of visnagin. However, visnagin (visnagidin) has been shown by Samaan to crystallize more like khellol-glucoside (khellinin). Small differences in methods of crystallization may produce marked changes in gross crystal patterns; however, the long-fibrous, bulky threads of visnagin are sufficiently distinctive of this compound to preclude its association with khellin and khellol-glucoside. Khellol-glucoside was recrystallized from absolute methanol and found to have a melting point of 176.2 to 177.0°. Rapid crystallization produced the star-like crystals of Fig. 1, *E*, while slower crystallization gave the opaque crystals of Fig. 1, *F*.

Solubility studies in 95% ethanol, after the method of Kunitz and Northrop (22), gave an indication of pure, single composition material in the case of natural khellin and visnagin. Khellol-glucoside purity was necessarily based on recrystallization producing a constant melting point, due to its limited solubility.

Tetraethyl ammonium bromide (Eastman Kodak No. 1516), used as the supporting electrolyte in the polarographic method, was purified by recrystallization from an ethanol-water mixture. The solvents, cyclohexane, chloroform, and ethanol used for the spectral survey, were of spectroscopic purity; while those solvents used for the polarographic studies required further purification to give a polarographic blank over the voltage range used.

The infrared measurements were determined from 2 to 12 μ with a Perkin-Elmer Model 21 instrument equipped with rock salt optics. Absorption data in the ultraviolet were obtained from a direct recording Cary spectrophotometer equipped with quartz optics and a Nester hydrogen discharge lamp.

A Sargent-Heyrovsky Model 12 photographically recording instrument was used in the polarographic study.

DISCUSSION

Infrared.—The infrared absorption spectra from 2 to 12 μ are shown in Figs. 2, 3, and 4. The spectra of khellin and visnagin were obtained in chloroform solution at a concentration of 100 mg./ml. of solvent. Over-compensation for solvent absorption may be noted in a portion of the spectrum from 2 to 5 μ. The infrared absorption spectrum of khellol-glucoside (Fig. 4) was obtained from a suspension of solid material in a paraffin base, since the glucoside is practically insoluble in chloroform. In Fig. 2, synthetic khellin is compared to the purified natural material. The complete matching of the curves confirms the identity of the two, and at the same time, verifies the structure given. In Fig. 3, a similar confirmation of the structure assigned to visnagin has been made by a comparison of the spectrum of the natural material to that of the material synthesized in our laboratories.

Table I lists in tabular form the absorption data in the region studied for each of the three compounds. The letter S refers to strongly absorbing bands, M to moderately strong bands, and W to those bands which are comparatively weak. The symbol ∽ signifies inflection points where complete resolution of the band has not taken place. The strong band at 6.00 μ, observed in all three compounds, may be assigned to the vibration of the conjugated carbonyl structure. This represents a shift from the unconjugated ketone absorption at 5.83 μ of 0.17 μ (49 cm.$^{-1}$), and from the simple conjugated ketone —C=C—C=O absorption at 5.90 μ— a shift of 0.10 μ (29 cm.$^{-1}$). Acetophenone has been reported (23) to have a conjugated ketonic band at 5.93 μ. The more extensive conjugation and resonance of khellin, visnagin, and khellol-glucoside have, therefore, shifted this band 16 cm.$^{-1}$ to the longer wave length. A similar correlation of the bands in the region of 6.15 μ to the conjugated —C=C—C=C— could be made.

Skeletal frequencies beyond 6.50 μ cannot be as readily related to molecular groups; however, the

TABLE I.—TABULATION OF INFRARED SPECTRAL DATA

Khellin, $\lambda_{max.}$ μ	Visnagin, $\lambda_{max.}$ μ	Khellol-Glucoside, $\lambda_{max.}$ μ
3.30 S	3.30 S	3.40–3.45
3.36 M	∽3.36	6.00 S
3.47 W	3.47 W	6.12 M
6.00 S	6.00 S	6.25 S
6.16 S	6.15 S	6.45 M
∽6.24	6.27 S	6.82 S
6.45 M	6.45 M	7.10 M
6.75 S	6.80 S	7.24 S
6.93 S	6.95 S	7.40 S
7.20 S	7.17 S	7.52 M
∽7.30	7.30 S	7.63 M
7.38 S	7.45 S	7.80 M
∽7.45	7.90 W	7.97 W
7.67 W	∽8.35	8.25 M
7.80 M	8.50 S	8.55 M
8.09 W	8.72 S	8.83 M
8.40 M	8.98 S	9.03 S
8.52 M	9.20 S	9.30 S
8.65 M	9.32 S	9.42 S
8.87 S	9.62 W	9.70 S
9.40 S	9.90 W	10.07 M
9.87 M	10.33 S	10.32 M
10.10 M	10.50 M	11.09 W
10.27 M	11.03 M	11.56 M
10.75 M	11.78 S	
11.35 W	12.00 M	
11.80 S		

[1] Samaan, in his studies, refers to khellin as visammin, visnagin as visnagidin, and khellol-glucoside as khellinin.

shifting of these bands has provided a convenient means for characterizing the three compounds. The bands best characterizing the khellin structure and which have been used for analytical determinations of this component are found at 8.87 and 10.75 μ. The visnagin content of mixtures may be determined by its absorption at 8.73, 8.98, and 11.03 μ. This difference in absorption between khellin and visnagin in the range of 8–10 μ is shown in Fig. 5.

Analytical estimation of khellin and visnagin in plant fractions have been made using this portion of their spectra. Absence of khellol-glucoside in

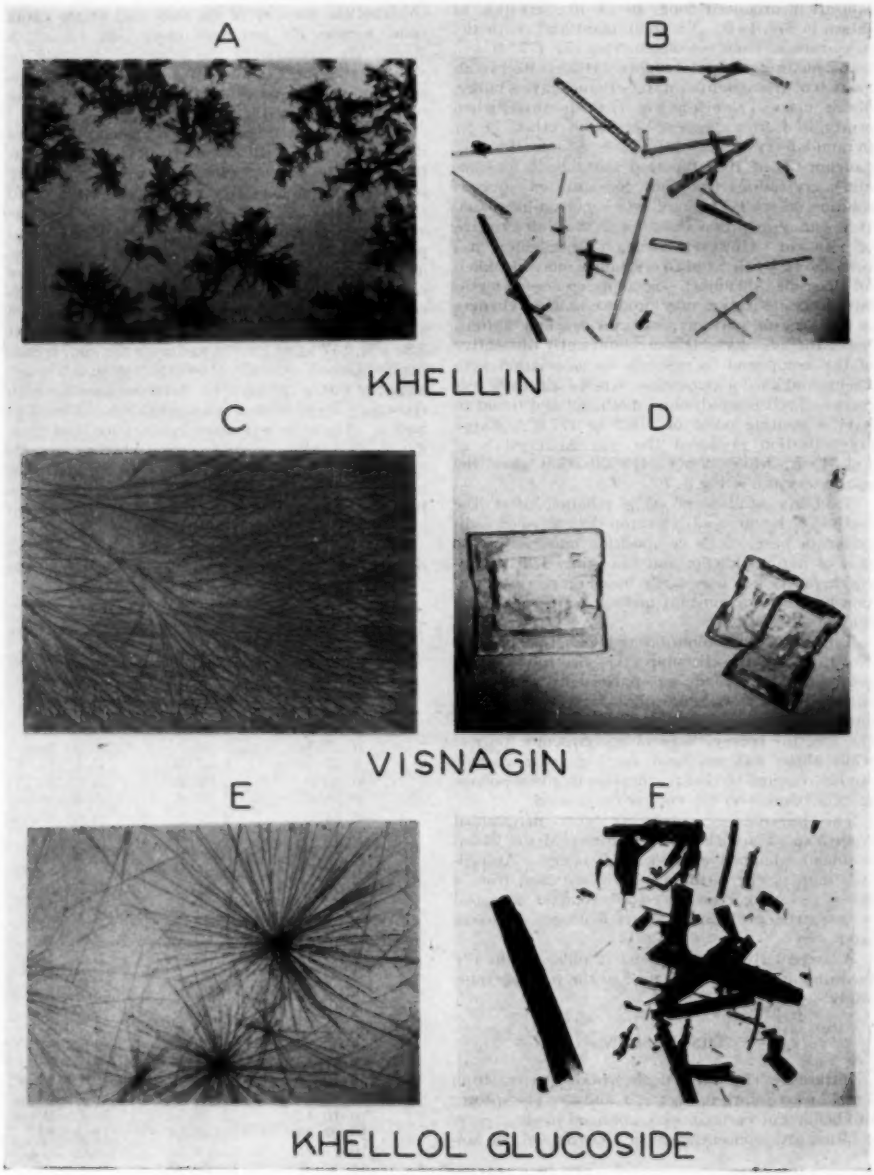


Fig. 1.—Photomicrographs of khellin, visnagin, and khellol-glucoside. 75 ×.

such plant fractions (due to solubility differences) insures no interference from this component.

The presence of both khellin and visnagin in extracts from the seeds of *Ammi visnaga* Lam. have been frequently observed. The absorption spectra of two typical plant fractions, *A* and *B*, are shown in Fig. 6 where absorption bands attributable to both khellin and visnagin may be observed.

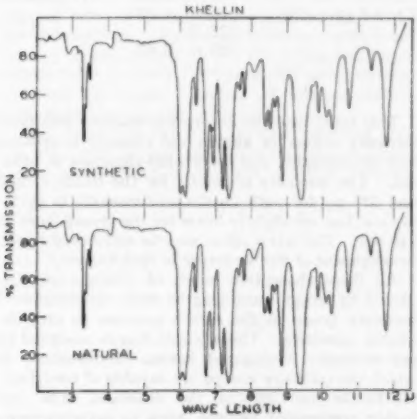


Fig. 2.—Infrared spectral curves of synthetic and natural khellin in chloroform.

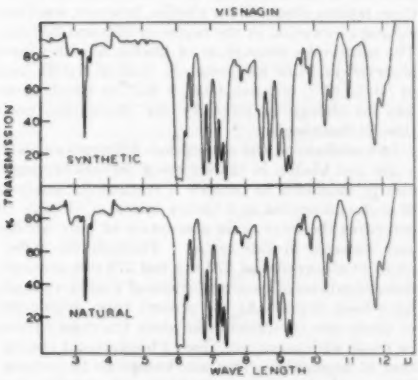


Fig. 3.—Infrared spectral curves of synthetic and natural visnagin in chloroform.

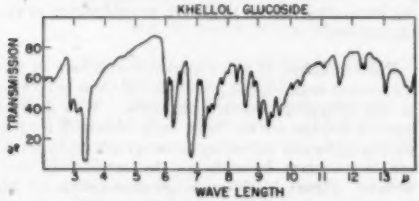


Fig. 4.—Infrared spectral curve of khellol-glucoside suspended in Nujol.

Ultraviolet.—The ultraviolet absorptions for these compounds from 210 to 380 mμ in 95% ethanol and cyclohexane are shown in Figs. 7, 8, and 9 at two concentrations. In accordance with the manufacturer's recommendation, absorbancies are most accurately determined in the optical density range from 1 to 2. Consequently, concentrations of the order of 4×10^{-5} *M*. were required for the strong bands, and 2×10^{-4} *M*. for the weaker bands. Slit widths in the region from 250 to 380 mμ were from 0.03 to 0.04 mm., while those from 230 to 250 mμ were from 0.04 to 0.08 mm., then gradually increasing to a maximum slit width of 1.07 mm. at 210 mμ.

The spectra are very similar, as would be expected, exhibiting bands in the regions of 250, 280, and 320 mμ for ethanol, and shifting to the shorter wave-length region when observed in the nonpolar solvent, cyclohexane. The ultraviolet spectra of

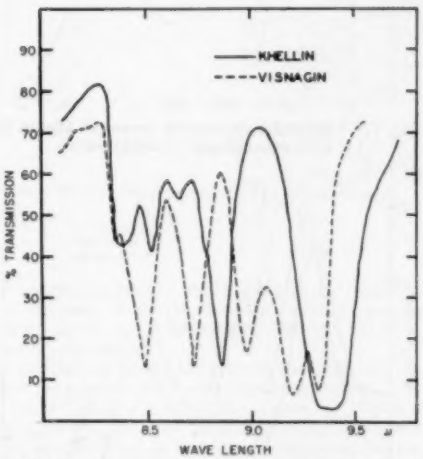


Fig. 5.—Comparison of the absorption bands of khellin and visnagin in the 8–10 μ region of the infrared.

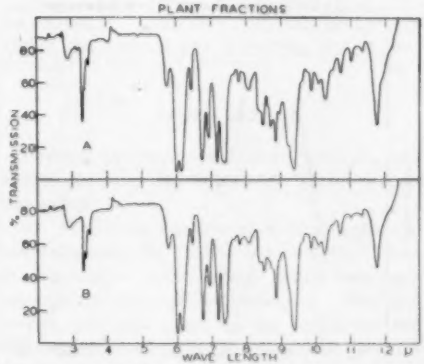


Fig. 6.—Comparison of the infrared spectra of two typical extracts from the seeds of *Ammi visnaga* Lam.

khellin and khellol-glucoside have been previously reported (20, 21) for identification and assay of these compounds, as well as a partial confirmation of the α-β unsaturated ketone structure. However, insufficient resolution had prevented the observation of the bands at 280 mμ. The ultraviolet absorption of visnagin had not been previously investigated.

The wave length of absorption maxima and the intensity of absorption are given in Table II.

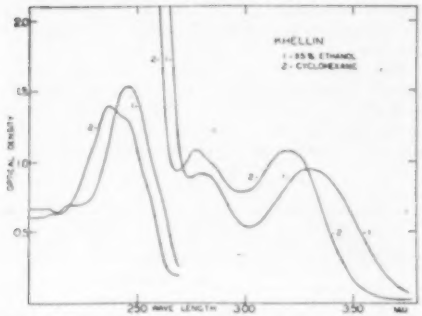


Fig. 7.—Ultraviolet absorption curves of khellin in (1) 95% ethanol and (2) cyclohexane.

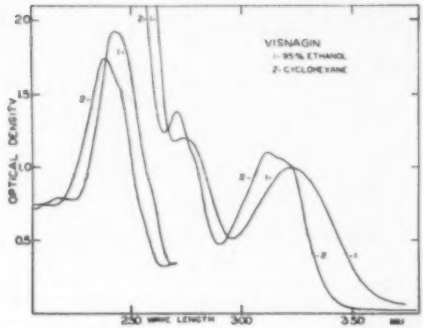


Fig. 8.—Ultraviolet absorption curves of visnagin in (1) 95% ethanol and (2) cyclohexane.

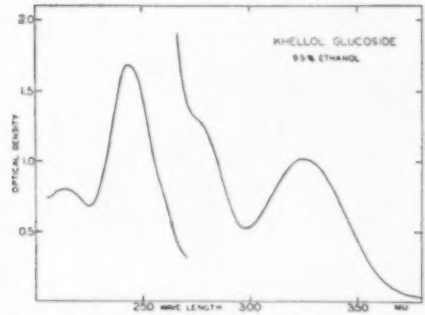


Fig. 9.—Ultraviolet absorption curves of khellol-glucoside in 95% ethanol.

TABLE II.—ULTRAVIOLET ABSORPTION DATA

Principle	95% Ethanol, $\lambda_{max.}$ mμ	log ϵ	Cyclohexane, $\lambda_{max.}$ mμ	log ϵ
Khellin	247.0	4.58	237.5	4.52
	281.0	3.66	278.0	3.72
	331.0	3.67	321.0	3.72
Visnagin	243.0	4.60	237.5	4.55
	275.0	3.78	270.0	3.84
	322.5	3.69	311.5	3.74
Khellol-glucoside	243.0	4.55		
	~277.5	...		
	325.0	3.67		

This table lists the absorption maxima and their intensity values for khellin and visnagin in ethanol and cyclohexane, and for khellol-glucoside in ethanol. The intensity is greater for the bands at 280 and 320 mμ for both khellin and visnagin in cyclohexane, but are slightly lower for the strong band at 240 mμ. The latter effect may be attributed to the development of fine structure in cyclohexane.

All three absorption bands of visnagin are displaced to longer wave lengths with the additional methoxy group in the eighth position to give the khellin structure. These bands may be assigned to the aromatic conjugated ketone chromophore in which the methoxy groups are capable of contributing to the resonance of the molecule. The less highly conjugated ketones, such as acetophenone, have similar absorption bands. Recently reported (23) absorption of acetophenone and hexadecanephenone in the ultraviolet showed absorption in the three regions observed for khellin, however, with less intense absorption in the region of 280 and 330 mμ. The ultraviolet absorption of khellin has also been observed in 50:50 95% ethanol, 0.05 *N* NaOH, and in 50:50 95% ethanol, 0.05 *N* HCl in which there was no change in intensity nor absorption positions in these media.

In cyclohexane the absorption differences of visnagin and khellin in the region of 280 mμ are sufficiently increased to provide a method for analysis of each component in a binary mixture. Figure 10 compares the ultraviolet absorption of pure khellin and visnagin in this region. Through the differences in absorption at 270 mμ and 278 mμ, accurate determinations of known mixtures of khellin-visnagin have been made. At the present time, estimation of these two components in plant fractions cannot be made with assurance, due to background absorption of impurities which are known to be present. This method is more sensitive and requires less material than the infrared for mixtures containing only khellin and visnagin. However, the infrared is to be preferred for analysis of materials containing impurities known to absorb in this region of the ultraviolet.

Polarography.—The conjugated structure of the chromones suggests the possibility of their reduction at the dropping mercury electrode. Well defined current-voltage curves have been obtained from a photographically recording polarograph using tetraethylammonium bromide as the supporting electrolyte. Figure 11 shows polarograms obtained for the three principles in a 1:1 water-95% ethanol medium. The half-wave potentials measured

against the reference mercury pool electrode with the aid of a Rubicon Potentiometer are as follows:

Khellin	1.547 volts
Visnagin	1.571 volts
Khellol-glucoside	
(*a*)	1.402 volts
(*b*)	1.592 volts

Wave steps were obtained in aqueous solutions and in solutions of propylene glycol, ethylene glycol, dioxane, and ethanol. However, the aqueous-ethanol medium proved most satisfactory for analytical purposes. Half-wave potentials were found to vary considerably with the *p*H of the medium as illustrated in Table III. The buffer solutions were prepared by proper blending of lithium acetate, lithium hydroxide, and acetic acid.

This dependence of the half-wave potential upon the *p*H of the medium may be attributed to participation of hydrogen ions in the electrode reaction.

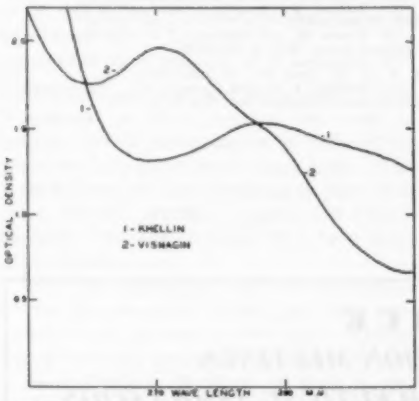


Fig. 10.—Comparison of the ultraviolet absorption of khellin and visnagin in cyclohexane in the region from 260 to 290 mμ.

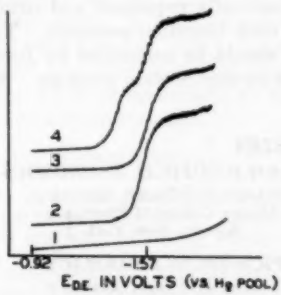


Fig. 11.—Polarographic curves of khellin, visnagin, and khellol-glucoside.

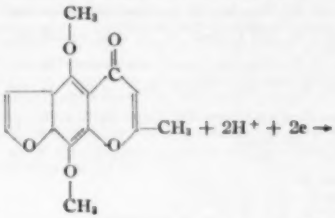
Solvent 1:1 water −95% ethanol. Supporting electrolyte −0.1 *M* $(C_2H_5)_4$ NBr. Temp. 25° C., sens. 1/5.

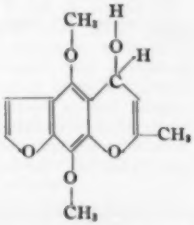
1. Blank
2. 2.7×10^{-4} *M* khellin
3. 2.5×10^{-4} *M* visnagin
4. 2.5×10^{-4} *M* khellol glucoside

TABLE III.—EFFECT OF *p*H ON THE HALF-WAVE POTENTIALS OF KHELLIN

*p*H	Concn. of Khellin	$E_{1/2}$ vs. S. C. E., Volts	Step Height, Mm.
6.04	2×10^{-4} *M*	−1.452	25.5
8.10	2×10^{-4} *M*	−1.674	34.4
10.02	2×10^{-4} *M*	−1.688	35.0

A suggested mechanism for the reduction of khellin at the dropping mercury electrode would involve the saturation of the carbonyl group.





A similar mechanism would apply to the reduction of visnagin; while the two-step reduction process found in the case of khellol-glucoside may not be as readily explained.

Neither the reversibility of the electrode reaction nor the reaction mechanism has been proved. The plot of log $i/(i_d - i)$ *vs.* $E_{d.e.}$ gives for khellin a straight line, the slope of which gives a value of 0.7 for *n*—the number of electrons involved in the electrode reaction. Such a value would indicate that the electrode reaction is irreversible (24).

The proportionality of the diffusion current to the concentration of khellin, visnagin, and khellol-glucoside provided a method for their quantitative determination. Qualitative differentiation between khellin and visnagin was impossible, due to their similarity in half-wave potentials.

SUMMARY

Physical properties of khellin, visnagin, and khellol-glucoside are reported. These properties include:

1. The infrared spectra of both synthetic and naturally occurring khellin and visnagin. These results confirm the structures which have been assigned to these active principles. This procedure has been useful for the estimation and differentiation of visnagin and khellin in plant fractions and pharmaceutical mixtures.

2. The ultraviolet absorption for khellin, visnagin, and khellol-glucoside. The khellin spectra is shifted to the longer wave lengths as a result of the presence of the positive auxochrome, —O—R, which is capable of resonating with the conjugated ketone structure. In cyclohexane this difference over visnagin is enough to provide a means of quantitatively determining khellin (or visnagin) in mixtures of these two components.

3. The polarographic characteristics of khellin, visnagin, and khellol-glucoside. The half-wave potentials at the dropping mercury electrode have been observed. Those for khellin and visnagin are very similar—a single wave step being obtained; whereas glucoside involves a two-step reduction. The diffusion current values have been useful for the quantitative estimation of combined khellin and visnagin.

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NOTICE

PAPERS FOR SECTION MEETINGS OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

The next annual meeting of the American Pharmaceutical Association will be held in Buffalo, New York, August 26 to September 1, 1951. Members planning to present papers before any Section during this meeting are earnestly requested and strongly urged to notify the appropriate Section secretary of their intention promptly. Titles and abstracts (not to exceed 250 words) of papers should be submitted by June 1, 1951 or preferably earlier, in order to insure a place on any Section program. Send titles and abstracts of papers to:

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Khellin. Part II. Methods for the Determination of Two Principles Isolated from *Ammi visnaga* Lam.*,†

By W. C. ELLENBOGEN, E. S. RUMP, P. A. GEARY, and M. BURKE‡, §

Since khellin, visnagin, and khellol-glucoside, isolated from the plant *Ammi visnaga* Lam., have been proved to be compounds of pharmaceutical interest, it was necessary to devise methods for their quantitative determination, either singly or in mixtures. Because the glucoside was easily removed from the samples tested, our work was limited to a study of khellin and visnagin. A review of the literature revealed two colorimetric methods, using potassium hydroxide or sulfuric acid to develop the color. It was found, however, that these two methods lacked reproducibility and failed to distinguish between khellin and visnagin. To solve this problem several new methods were developed using ultraviolet, infrared, and polarographic techniques. The ultraviolet and polarographic methods were found to give reproducible results on the determination of khellin, visnagin, or mixtures of both, when the results were reported in terms of a standard compound. It was also found possible to determine the ratio of khellin to visnagin in mixtures of the two by means of infrared absorption, and by use of these data, to translate the ultraviolet or polarographic results into absolute quantities of each component. Both methods were satisfactory, but the ultraviolet was preferred because of its brevity and simplicity.

IN THE PAST years a number of publications have appeared in which mention was made of methods for the determination of some of the principles of the plant *Ammi visnaga* Lam. Most of the interest has been displayed in three principles, namely: khellin, visnagin, and khellol-glucoside, formulas of which have been given in the preceding paper.

None of the existing methods were satisfactory for the determination of the individual compounds in the presence of each other. Khellol-glucoside is easily removed from samples because of its insolubility. However, visnagin is very similar to khellin in its chemical and physical properties, so that it was desirable to determine the amount of either in the presence of the other, or to determine the total amount present, in terms of the absolute amounts present reported as khellin plus visnagin. This communication is, therefore, concerned with a discussion and comparison of some of the methods for the determination of khellin and visnagin.

EXPERIMENTAL

Alkaline Hydrolysis Colorimetric Method.—The method that has been used most in the past appears to be one originally devised by Fahmy and El-Keiy (1). It depends on the visual estimation of a rather stable, pinkish-red color that develops when an aqueous solution of khellin is in contact with solid sodium hydroxide. Anrep, *et al.* (2–5), modified this method by using a saturated solution of potassium hydroxide. The color that developed was then measured either visually or with a photoelectric colorimeter. When tried in our laboratories, this method did not appear to be satisfactory as a precise method for the quantitative estimation of khellin and related compounds. Modifications of this method were made as follows:

One milliliter of an aqueous solution containing 0.2 mg. of the sample per ml. was pipetted into a colorimeter tube. Five milliliters of 19 *M* sodium hydroxide solution was then added to the colorimeter tube from a pipette. The tube was stoppered with a rubber stopper (previously boiled with alkali and washed), immediately shaken for five seconds, and centrifuged at 2,000 r. p. m. for five minutes to remove any turbidity. The absorbancy was read on a Klett-Summerson colorimeter between fifteen and twenty-five minutes after the addition of the alkali, using filter No. 54. The reading was corrected for a reagent blank and compared against a standard sample of crystalline khellin treated in the same manner. It was found that the color itself was relatively stable but developed rather slowly, and the readings had to be taken at a definite interval after mixing the reactants. Furthermore, the intensity of the color was dependent upon the exact manner of adding and mixing the reactants. The alkali had to be protected from carbon dioxide, which produced a turbidity and made the measurement difficult, even after centrifugation. The colorimeter tubes were affected by the strong alkali and frequently changed their calibration. All of these variables resulted in data which could not readily be reproduced (see Table I). Furthermore, it was found that visnagin gave the same color as khellin although the intensity differed. This made it impossible to differentiate between khellin and visnagin and to estimate the amount of either of the components in mixtures of both.

Acid Hydrolysis Colorimetric Method.—A second colorimetric method was published in 1949 by Fahmy, Badran, and Messeid (6, 7) based on the

* Received March 20, 1951, from the Research Division of Smith, Kline and French Laboratories, Philadelphia, Pa.
† Presented to the Pharmacy Subsection of the American Association for the Advancement of Science, Cleveland meeting, December 28, 1950.
‡ Research Division, Smith, Kline and French Laboratories, Philadelphia, Pa.
§ The authors are indebted to Mr. John C. Souder, Smith, Kline and French Laboratories, for suggestions and assistance in the infrared work.

TABLE I.—KHELLIN AND VISNAGIN (COMPARED BY SEVERAL METHODS OF ANALYSIS)

	Theoretical, %		% Total Recovery in Terms of Pure Khellin Standard			% Total Recovery Corrected, % Khellin Plus % Visnagin	
Sample No.	Khellin	Visnagin	NaOH Method	H_2SO_4 Method	Ultraviolet Method	Ultraviolet Method	Polarographic Method
1	100	...	...	...	Std.	...	...
2	...	100	60–70	70–75	114	...	...
3[a]	80.75	19.25	70.9	96.7	101.3	99.47	98.5
			94.5	94.5	101.6	99.77	100.0
			68.7	94.7	100.0	98.2	...
			80.5	...	...	...	...
4[a]	69.02	30.98	77.6	92.1	102.1	99.24	100.9
			76.4	92.1	101.7	98.8	100.9
			64.0	92.6	101.4	98.6	...
			64.6	...	...	...	...
5[a]	60.13	39.87	79.4	89.6	101.8	98.13	99.3
			83.1	88.6	102.2	98.5	...
			74.1	87.5	102.7	99.0	...
			70.3	...	...	...	...
6[b]	...	...	88.3	74.64	82.4	80.59	80.9
			79.2	71.51	83.2	80.40	81.0
			77.4	74.12	82.8	80.97	...
			87.5	...	...	...	...
			81.7	...	...	...	...
7[b]	...	...	50.9	66.95	72.26	70.52	70.2
			51.8	68.56	72.29	70.56	68.6
			59.7	66.19	71.86	70.14	..

[a] Synthetic mixtures.
[b] Natural extracts.

reaction of khellin and related compounds with sulfuric acid. This method was found to be better than the sodium or potassium hydroxide methods when run as follows:

A solution was prepared to contain 0.2 mg. of sample per ml. A 2.50-ml. aliquot of this solution was pipetted into a 10-ml. colorimeter tube and 10.00 ml. of 10 *N* sulfuric acid added from a pipette or a burette. The solutions were mixed by inverting, allowed to stand for five minutes, and the absorbancy read on a colorimeter using a No. 42 blue filter. The reading was corrected for a reagent blank and compared against the absorbancy of a standard solution of crystalline khellin run in exactly the same manner.

The results obtained by this method (see Table I) were found to be a great improvement over those derived from the sodium hydroxide method. This was probably due to better control of the reaction. A definite concentration of acid was used and the khellin sulfate responsible for the color could be isolated in a pure form and used as a standard if desired. Results were much more reproducible. However, it was found once again that visnagin produced the same color as khellin, and although the color from visnagin differed in intensity from that of khellin, the similarity of the two makes the estimation of khellin or visnagin in the presence of each other impossible. Furthermore, in the assay of tablets there is a possibility of excipient substances producing a yellow color with sulfuric acid and thus necessitating the use of a tablet blank.

Ultraviolet Absorbancy Method.—It was thought that a better method for the estimation of khellin and related compounds could be devised, using physical methods. Samaan, *et al.* (8), had published information on the ultraviolet absorption of khellin, and a great deal of work on the physical properties of khellin and other principles of *Ammi visnaga* was done in our laboratories, as reported in the preceding paper. Ultraviolet absorption methods were first investigated, using a Beckman Model DU Spectrophotometer. It was found that pure khellin in 95% ethyl alcohol, U. S. P. had a maximum absorption at 247 mμ, but that its extinction coefficient ($E\ ^{1\%}_{1\ cm.}$) was 1,460–1,480 rather than 1,600, as reported by Samaan. Although there was no separation of the absorption maxima of khellin and visnagin in mixtures, pure khellin and pharmaceutical products containing khellin were analyzed for khellin or khellin plus visnagin in the following manner (results being reported in terms of khellin):

One hundred milligrams of khellin, or pharmaceutical products containing 100 mg. of khellin, were dissolved in 200 ml. of 95% ethyl alcohol, U. S. P., shaken well and allowed to stand for one hour. The solution was filtered, if necessary, and 1.0 ml. diluted to 200 ml. in a volumetric flask, with alcohol. The final solution contained about 2.5 μg./ml. The absorbancy was read on a Beckman Model DU Spectrophotometer in a 1-cm. quartz cell at 247 and 274 mμ. The absorbancy at 274 mμ was subtracted from the absorbancy at 247 mμ and compared against the differential absorbancy obtained by treating a standard solution of crystalline khellin in the same manner. These results were reproducible to ±2%, as indicated in Table I.

To eliminate possible effects of a background absorbancy in this determination, a difference of absorbancies was used for the comparison rather than the absorbancy at any one wave length. It was found that this method was much superior to the preceding methods, by virtue of its reproducibility and ease of operation.

A second ultraviolet method was devised when it was found that the use of cyclohexane as a solvent, in more concentrated solutions, produced a separation of the absorption maxima of khellin and vis-

TABLE II.—KHELLIN AND VISNAGIN (COMPARED BY ULTRAVIOLET AND INFRARED ANALYSIS)

Sample No.	Theoretical		Ultraviolet[a] (Cyclohexane) Method		Infrared Method	
	Khellin, %	Visnagin, %	Khellin, %	Visnagin, %	Khellin, %	Visnagin, %
1	100	0	100	0	100	0
2	...	100	0	100.0	0	100
3[a]	80.8	19.2	79.2	20.9	79	21
4[a]	69.02	30.98	69.1	30.9	66	34
5[a]	60.13	39.87	59.6	40.4	56	44
6[b]	...	...	...	...	76	24
7[b]	...	...	...	...	81	19

[a] Synthetic mixtures.
[b] Natural plant extracts.
[c] Total recovery, 98.5 to 100%.

nagin in the region of 260–290 mμ. This method was run as follows:

The sample was dissolved in cyclohexane, filtered if necessary, and diluted until a concentration of 20 μg./ml. was obtained. The absorbancies were read on a Beckman Model DU Ultraviolet Spectrophotometer at 271 and 278 mμ. The density values were then inserted in the following formula, which was derived according to the method of simultaneous equations:

$$\text{Concn. of khellin (Gm./100 ml.)} = \frac{[A_{271} \times E_{v-278}] - [E_{v-271} \times A_{278}]}{[E_{k-271} \times E_{v-278}] - [E_{v-271} \times E_{k-278}]}$$

$$\text{Concn. of visnagin (Gm./100 ml.)} = \frac{[A_{278} \times E_{k-271}] - [E_{k-278} \times A_{271}]}{[E_{k-271} \times E_{v-278}] - [E_{v-271} \times E_{k-278}]}$$

where

A_{278} = absorbancy at 278 mμ.
A_{271} = absorbancy at 271 mμ.
$E_{k-278} = E^{1\%}_{1\text{ cm.}}$ of khellin at 278 mμ.
$E_{k-271} = E^{1\%}_{1\text{ cm.}}$ of khellin at 271 mμ.
$E_{v-278} = E^{1\%}_{1\text{ cm.}}$ of visnagin at 278 mμ.
$E_{v-271} = E^{1\%}_{1\text{ cm.}}$ of visnagin at 271 mμ.

Results are shown in Table II. Obviously, this method is better than the first ultraviolet method by virtue of its ability to give an absolute analysis of the separate components in the mixture. However, work done to date has indicated that the background encountered in crude extracts has eliminated the use of this method in these cases.

Polarographic Methods.—The polarographic method used for the determination of pure khellin or pharmaceutical products containing khellin was as follows:

A Sargent-Heyrovsky Model XII polarograph was used at a sensitivity of 5 (0.0250 ma./mm.). The $m^{2/3}t^{1/6}$ value of the capillary used was 1.500 $mg.^{2/3}$ $seconds^{-1/2}$ measured on open circuit at the pressure of mercury used throughout (58.5 cm.). Temperature was maintained at 25° ± 0.1°. The cell employed was an H-type. Each half had approximately 12-ml. capacity. One-half, separated from the sample cell by a sintered glass disk, was a saturated calomel anode.

The sample was dissolved in distilled water to a concentration of approximately 100 μg. of khellin per ml., using a hot water bath to aid solution. To an 8.00-ml. aliquot of this solution was added 2.00 ml. of 0.1 *N* tetraethyl ammonium hydroxide, prepared from recrystallized tetraethyl ammonium bromide, sodium-free silver oxide and water. This solution was poured into a polarographic cell and oxygen-free nitrogen passed through the solution for ten minutes. The polarogram was then promptly obtained over the range of approximately −0.9 to −2.0 volt, with nitrogen passing over the solution during the run. The step-height obtained with the sample was referred to a standard working curve of step-height *vs.* khellin concentration.

The polarographic wave due to reduction of khellin in this medium is well defined, gives reproducible values (±2%), and is proportional to the concentration of khellin. There is a tendency for the khellin to decompose in this basic medium, so all samples must be run approximately the same time after the addition of the base. (No difficulty was encountered with a series of samples where the time of starting the polarogram varied from eleven to fourteen minutes after the addition of the base.)

Under the above conditions, a standard khellin sample gave a step-height of 0.677 mm./μg. of khellin per ml. The half-wave potential was approximately 1.5 volts *vs.* the S. C. E.

Results obtained here (see Table I) were in close agreement with the first spectrophotometric results. This method also gave total amounts of khellin and visnagin in terms of khellin and, as in the case of the ultraviolet method, could not distinguish between the two compounds. The advantage of the ultraviolet methods over the polarographic is the ease with which the former can be carried out and the ability of the second ultraviolet method to give separate analyses in various mixtures of the two.

Infrared Methods.—One of the disadvantages of most of the methods mentioned thus far was their inability to distinguish between khellin and visnagin. Using the potassium or sodium hydroxide method, visnagin will give 66% of the color intensity of an equal weight of khellin, and with the sulfate method about 70–75% intensity of the color from khellin. Using the first ultraviolet spectrophotometric method described here, the optical density of visnagin was approximately 112–114% of that obtained from an equal weight of khellin, corresponding to the ratio of their molecular weights. The results from the polarographic method are similar to those from the ultraviolet method. To get the ratio of one component to another (before the second ultraviolet method was devised), use was made of the infrared absorption spectra of chloroformic solutions of the two compounds, and of the samples

in question. These spectra were obtained by the following method:

The assay was performed on a Model 12C Perkin-Elmer spectrometer using NaCl optics. The cell thickness was 0.1 mm. and the "cell in - cell out" technique employed. The latter necessitated calibration of the sample cell at each wave length with the solvent, $CHCl_3$, in both cells. The wave lengths selected for assay were 11.03, 10.93, 9.05, and 8.87 μ. The difference in absorbancy between 11.03, and 10.93 μ was used as a measure of the visnagin content and the difference between 8.87 and 9.05 μ indicated the amount of khellin. Standard curves of khellin and visnagin were prepared using concentrations up to 100 mg./ml. Both sets of wave lengths were used for both compounds.

In the analysis, approximately 1.0 Gm. was dissolved in $CHCl_3$ in a 10-ml. volumetric flask and brought to volume. The absorbancy at the selected wave lengths was obtained and the difference between the pairs determined. Using the graphic method of successive approximations (9), the amounts of khellin and visnagin were found and the ratio of khellin to visnagin calculated. This method was found to be reproducible to $\pm 2\%$.

Table II shows the ratios of khellin to visnagin as determined by the infrared method. These data may also be used to correct the amounts of total khellin plus visnagin, as obtained by the first ultraviolet or polarographic methods, correcting for the differences in molecular weights and the subsequent difference in absorption or step-height. The formula for this correction is as follows:

$$\text{Observed \% khellin plus visnagin in terms of khellin} \times \left[\frac{1 + V/K}{1 + \frac{A \times V/K}{B}}\right] = \text{\% khellin plus \% visnagin}$$

where

V/K = ratio of visnagin to khellin, as determined by infrared.

A = step-height or absorption of standard visnagin per unit concentration.

B = step-height or absorption of standard khellin per unit concentration.

The results of this correction may be seen in Table I.

SUMMARY

Five methods for the determination of khellin and khellin-like principles are discussed:

1. The potassium or sodium hydroxide colorimetric methods are not satisfactory because of the many variables and the corresponding lack of reproducibility.

2. The modified sulfuric acid colorimetric method is a great improvement over the first procedure by virtue of its greater reproducibility but does not give true values. Furthermore, the possibility of error from excipient materials is still very great.

3. Ultraviolet absorption technique presents a very convenient and reproducible method for the determination of khellin and khellin-like principles. In its final form, it is capable of permitting analysis of the individual components in mixtures of khellin and visnagin except for those occurring in crude extracts.

4. The polarographic method gives results equivalent to those from the ultraviolet determination but the procedure is less convenient.

5. Infrared absorption procedure may be used to obtain ratios of one component to another but the method as described is not satisfactory for absolute quantitative results, and is not as accurate as the second ultraviolet method when used with pure materials. However, new infrared methods now being developed using a double-beam instrument indicates very good accuracy and convenience.

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The Infrared Determination of Quinine and Strychnine in N. F. Preparations*

By WILLIAM H. WASHBURN and ELMER O. KRUEGER†

An infrared method for the simultaneous determination of quinine and strychnine in Elixir I. Q. and S. Phosphates is reported. A method for the separate determination of quinine by fluorometric means and strychnine by infrared in Elixir I. Q. and S. is also described. These procedures were found to be more accurate for small amounts of strychnine than previously described methods.

THE NATIONAL Formulary does not give a method of analysis for quinine or strychnine in Elixir I. Q. and S., or Elixir I. Q. and S. Phosphates. The procedure of determining the total combined quinine and strychine by gravimetric methods without separating the extract into its components has been found to be inadequate. This is due to the fact that the quantity of strychnine present in I. Q. and S. Phosphates is $^1/_{30}$ that of quinine, and in Elixir I. Q. and S., it is $^1/_{50}$ that of quinine.

Several methods for the determination of strychnine in elixir preparations have been reported. Among the more recent are a titrimetric determination by Brownlee (1), a colorimetric method by Chambon (2), and a polarographic method by Reimers (3). More notable was a method developed by Herd (4) in which N. F. preparations of quinine and strychnine in mixture are separated and each determined titrimetrically. This method, while greatly increasing the element of accuracy, requires a rather lengthy extraction procedure to accomplish separation of the components. The Association of Official Agricultural Chemists suggests a tentative gravimetric method for the separate determination of strychnine and quinine in liquid preparations (5).

In our laboratories a method was developed whereby quinine and strychnine are extracted from the Elixir I. Q. and S. Phosphates as one residue, and then determined simultaneously by measurement of their infrared absorption. For the Elixir I. Q. and S., a fluorometric method for quinine and an infrared method for strychnine were developed. This was necessary because the 50:1 relationship of quinine and strychnine will not permit a simultaneous infrared determination of each component.

EXPERIMENTAL

Instrument.—The spectrometer used in performing this work was a Perkin-Elmer model 12B automatic recording spectrometer equipped with a sodium chloride prism, windows, and absorption cells. A glass shutter was used to minimize the effects of stray light and a slit drive[1] was used in scanning the pure standards to permit a continuous spectrum. The cells employed were approximately 0.50 mm. thick, and similar in design to a type described by Tufts and Kimball (6). Spacers were made of polyethylene. No effort was made to match the cells perfectly, but the difference in optical density between blank and sample cells at the given wave lengths was determined and checked frequently.

Infrared Absorption Curves.—Solutions of U. S. P. strychnine and quinine of suitable concentration (about 20 mg./cc.) were prepared using chloroform, reagent grade, as solvent. Using a 0.50-mm. cell, the infrared absorption of each was recorded from 2.8 to 11.8 μ, and the spectra of each examined for possible analytical bands. As can be seen from the curves in Fig. 1, strychnine has a strong band at 6.06 μ, and quinine a strong band at 6.2 μ.

Working Curves.—From preliminary experimentation it was found that strychnine in the range of 3–5 mg./cc. of $CHCl_3$ gave sufficient absorption at 6.06 μ to permit an accurate determination. Concentrations of strychnine in the range of 1–5 mg./cc. of chloroform were therefore prepared, and spot readings taken at 6.2 and 6.06 μ. Readings were taken following the technique of Daasch (7). Samples were prepared by extracting strychnine phosphate from alkaline solution with chloroform.

In a similar manner, solutions of quinine base, previously converted from the phosphate, were prepared in the range from 10–80 mg./cc. of chloroform, and the absorption noted at 6.2 and at 6.06 μ. Optical densities were then plotted against concentration for each of the components at 6.2 and 6.06 μ. As can be seen in Fig. 2, the relationship was found to be linear.

In Fig. 3 are the working curves prepared in a similar manner for strychnine sulfate and quinine hydrochloride for Elixir I. Q. and S.

Infrared Method for Quinine and Strychnine in I. Q. and S. Phosphates.—Because of the 1:20 relationship of strychnine to quinine in the I. Q. and S. Phosphates preparation, both components cannot

* Received December 5, 1950, from the Control Department, Abbott Laboratories, North Chicago, Ill.

† The authors gratefully acknowledge the helpful suggestions from F. L. Ferger of the Control Department, Abbott Laboratories, which led to the successful extraction of quinine and strychnine from the elixirs.

[1] The specifications for the slit drive were kindly furnished by Mr. Jonas Carol, Food and Drug Administration, Washington, D. C.

be determined accurately on a single dilution. Rather, it is necessary to observe the optical density at 6.06 μ when the concentration of strychnine is about 4 mg./cc. and then dilute to bring the concentration of quinine down to the range of 25 to 30 mg./cc. and observe the optical density at 6.2 μ.

Transfer 30 cc. of sample to a 250-cc. beaker and place on a steam bath for fifteen minutes, or until reduced to a volume of approximately 25 cc. Transfer quantitatively to a 250-cc. separatory funnel and add dilute metaphosphoric acid until solution is acid to Alkacid paper. Extract gently with four 25-cc. portions of chloroform and discard chloroform. Add 2 *N* sodium hydroxide to aqueous portion until alkaline to Alkacid paper. Extract vigorously with four 15-cc. portions of chloroform, and combine chloroform extracts in a second 250-cc. separatory funnel. Wash with 10 cc. of 0.1 *N* sodium hydroxide, and using a 25-cc. flask, evaporate to dryness under a stream of nitrogen. Dry for two hours in a vacuum desiccator.

Dissolve residue in exactly 2 cc. of chloroform. Transfer 1 cc. of this solution to a small test tube, add 2 cc. of chloroform, and stopper tightly. Fill blank cell with chloroform and adjust slit for full scale deflection at 6.06 μ. Fill the sample cell with concentrated solution prepared above and observe the optical density. With blank cell in place, adjust slit for full-scale deflection at 6.2 μ. Fill sample cell with dilute solution prepared above and observe the optical density at 6.2 μ. This operation should be done as rapidly as possible to prevent evaporation.

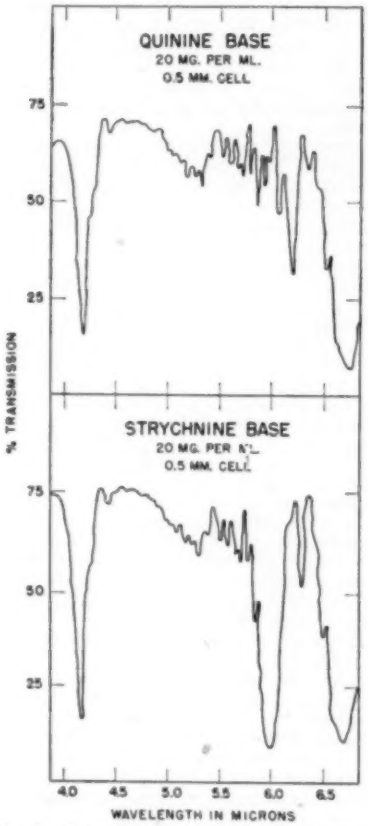

Fig. 1.—The absorption spectra of quinine and strychnine in chloroform solution. (The strong bands at 4.2 and 6.7 μ are due to chloroform.)

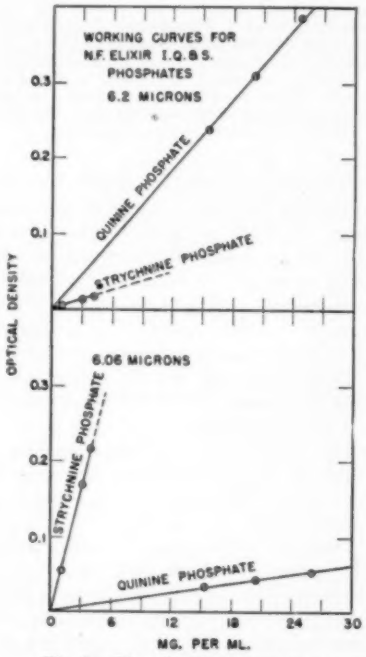

Fig. 2.—Plot of optical density *vs.* concentration for quinine phosphate and strychnine phosphate at 6.2 and 6.06 μ.

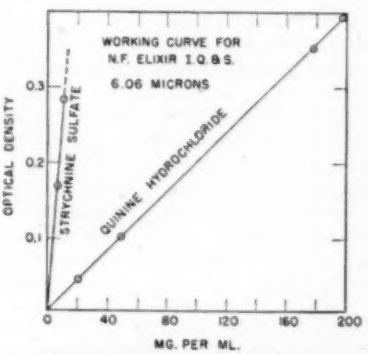

Fig. 3.—Plot of optical density *vs.* concentration for quinine hydrochloride and strychnine sulfate at 6.06 μ.

The concentration of each component is computed by a two-component graphical calculation, allowing for the fact that the solution observed for the strychnine band at 6.06 μ is three times as concentrated as that observed at 6.2 μ for the quinine band. In Table I are listed a series of results using this method.

TABLE I.—RESULTS OF ANALYSES ON THREE COMMERCIAL LIQUIDS BY THE PROPOSED METHOD FOR ELIXIR I. Q. AND S. PHOSPHATES

Sample No.	Quinine Phos., Mg./Cc. Theory	Quinine Phos., Mg./Cc. Recovered	Strych. Phos., Mg./Cc. Theory	Strych. Phos., Mg./Cc. Recovered
1	5.0	5.18	0.250	0.253
2	5.0	5.12	0.250	0.244
3	5.0	4.92	0.250	0.254

Fluorometric Method for Quinine in Elixir I. Q. and S.—

Apparatus and Reagents.—

1. 0.1 *N* sulfuric acid: dilute 2.8 cc. of concentrated H_2SO_4 to 1,000 cc. with water.
2. Standard quinine hydrochloride solutions:
   (*a*) Stock standard solution: prepare by dissolving 80 mg. of quinine hydrochloride in 1,000 cc. of 0.1 *N* H_2SO_4.
   (*b*) Working standard solution: dilute 2 cc. of the above stock standard solution to 500 cc. with 0.1 *N* H_2SO_4.
3. Instrument: Coleman Fluorometer equipped with voltage regulator and PC-1 and B_1 filters.

Transfer 2 cc. of the Elixir I. Q. and S. to a 1,000-cc. volumetric flask and make up to volume with 0.1 *N* H_2SO_4. Transfer 2 cc. of this solution to a 500-cc. volumetric flask and make up to volume with 0.1 *N* H_2SO_4. Transfer a sufficient quantity of the working standard solution to a cuvette and adjust the instrument to 70. Immediately read the deflection produced by the fluorescence of the test solution. After the test solution is read, the standard sample is returned to the instrument and the reading checked to see that no change from the initial setting has occurred. Should this reading vary from the initial setting, the instrument should be readjusted with the quinine working standard.

Calculation.—

$$\frac{\text{Reading of test solution}}{70} \times 8.0 =$$

Gm. quinine hydrochloride per 1,000 cc.

Infrared Method for Strychnine in I. Q. and S.— Transfer 25 cc. of sample to a 250-cc. beaker and heat on a steam bath for fifteen minutes or until reduced to a volume of approximately 20 cc. Transfer quantitatively to a 250-cc. separatory funnel and add 5 cc. of 5% sulfuric acid. Extract gently with four 25-cc. portions of chloroform and discard chloroform. Add 2 *N* sodium hydroxide to the aqueous solution until alkaline to Alkacid paper. Extract vigorously with four 15-cc. portions of chloroform, and collect the combined chloroform extracts in a second 250-cc. separatory funnel. Wash with 10 cc. of 0.1 *N* sodium hydroxide. Using a 25-cc. Erlenmeyer flask, evaporate to dryness on a steam bath with the aid of a stream of nitrogen.

Dissolve the residue in exactly 1 cc. of chloroform and observe the optical density of the sample solution at 6.06 μ. Apply correction for absorption of quinine hydrochloride as determined fluorometrically, refer corrected optical density to working curve, and determine concentration of strychnine sulfate.

A simultaneous method for strychnine and quinine was attempted for Elixir I. Q. and S. using the same approach as that used in Elixir I. Q. and S. phosphates. The 1:50 relationship of strychnine to quinine, however, proved too great to allow an accurate analysis. Therefore the above method was developed. In Table II are listed results by this method.

TABLE II.—RESULTS OF ANALYSES ON THREE COMMERCIAL LIQUIDS BY THE PROPOSED METHOD FOR ELIXIR I. Q. AND S.

Sample No.	Quinine HCl, Mg./Cc. Theory	Quinine HCl, Mg./Cc. Recovered	Strych. Sulf., Mg./Cc. Theory	Strych. Sulf., Mg./Cc. Recovered
1	8.0	7.50	0.175	0.173
2	8.0	8.10	0.175	0.172
3	8.0	8.01	0.175	0.151

In our laboratories we have applied the infrared method for determination of strychnine to several other nonofficial elixirs, and for the determination of strychnine when present in mixture with aspirin, phenacetin, and camphor.

SUMMARY AND CONCLUSIONS

1. An infrared method has been developed for the simultaneous determination of quinine phosphate and strychnine phosphate in N. F. Elixir I. Q. and S. Phosphates.

2. A method has been developed for the determination of quinine hydrochloride by fluorometric means and strychnine sulfate by infrared in N. F. Elixir I. Q. and S.

3. The procedures are simple to perform.

4. Reproducibility is in the range of ±2.5 per cent.

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The Effect of Added Water on the Stability at 50° of Hydrogen Peroxide-Glycerol Solutions With and Without Oxine*

By ETHAN ALLAN BROWN and WILFRED KRABEK

At 50°, hydrogen peroxide-glycerol solutions, containing 8-hydroxyquinoline, show an increased stability with increasing water content. The presence of oxine markedly increases the stability of hydrogen peroxide-glycerol solutions over that of solutions without oxine. Hydrogen peroxide solutions, without oxine, show a minimum stability with the commercial C. P. grade of glycerol without added water, and an increase in stability with amounts of added water up to 70 per cent. Anhydrous glycerol showed a stabilizing action somewhat better than the commercial grade, but was less effective than a solution containing 70 per cent added water, particularly as the experiment progressed.

GLYCEROL was suggested as a stabilizer for aqueous solutions of hydrogen peroxide by Kingzett (1, 2), who added 2.5 per cent of the solution. Brown, *et al.* (3), have reported on the stabilizing action of commercial C. P. grades of glycerol when used as a solvent for urea peroxide. The present paper reports the effect of varying amounts of added water on the stability at 50° of hydrogen peroxide-glycerol solutions, with and without oxine (8-hydroxyquinoline).

EXPERIMENTAL

With the exception of the anhydrous solution, which was made with anhydrous glycerol, said to contain 0.01% water, the solutions were all made with the same lots of constituents.

To make the solutions, the amount of water to be added was placed in a tared bottle, and glycerol was added to bring the weight up to 350 Gm. total. After thorough mixing, portions of 100 and 200 Gm. were weighed out, respectively, into 4-oz. and 8-oz. bottles with plastic caps. The bottles were of amber glass. These amounts filled the bottles to approximately the same extent.

To each 8-oz. bottle was added 0.2 Gm. of oxine, the mixture being thoroughly shaken and allowed to stand overnight, with occasional shaking during working hours. The larger amount of solution was taken for the solution containing oxine, since it was anticipated that this solution would be more stable and require more titrations.

To each 4-oz. bottle (100 Gm. of solution) was then added 1.2 ml. of Becco 90% hydrogen peroxide, and to each 8-oz. bottle (200 Gm. of solution) was added 2.4 ml. of 90% hydrogen peroxide. The solutions were then thoroughly mixed and individually titrated by standardized iodine-thiosulfate method, reported by Brown, *et al.* (4).

The solutions were then stored at 50° in an Elconap oven and aliquot samples titrated at the intervals, and with the results indicated in Tables I and II. Selected values have been plotted, as shown in Fig. 1.

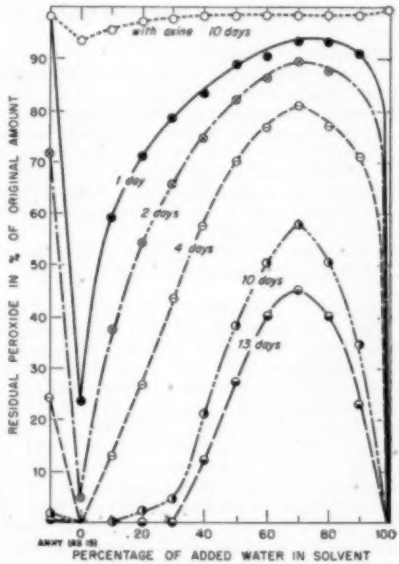

Fig. 1.—Effect of added water on stability of hydrogen peroxide in glycerol at 50°.

It is interesting to note from Fig. 2 that the commercial C. P. grade of glycerol (95%) gives solutions of the least stability, both with and without oxine. This is not observed with urea peroxide, where a minimum is obtained with about 30% added water. After one day, the aqueous solution of hydrogen peroxide has almost 10% more residual peroxide than the solution made with 95% glycerol. A low peroxide content (equivalent to 0.03 ml. of 0.1 *N* thiosulfate) frequently persists for months at 50°, in the absence of glycerol, while in the presence of glycerol (95%) the peroxide content goes quite rapidly to zero. This would seem to indicate that when the glycerol solution begins to oxidize, the products favor the decomposition of the residual peroxide.

With increasing lengths of time, the solutions without oxine show a maximum stability at an

* Received November 13, 1950, from the Medical Department, Tufts Medical School, and The Asthma Research Foundation, Inc., Boston, Mass.

TABLE I.—EFFECT OF ADDED WATER ON STABILITY AT 50° OF H_2O_2 GLYCEROL SOLUTIONS

Anhydrous			"As Is"			10% Water[a]			20% Water		
Days	Thio., Ml.	Resid. Perox., %	Days	Thio., Ml.	Resid. Perox., %	Days	Thio., Ml.	Resid. Perox., %	Days	Thio., Ml.	Resid. Perox., %
0	22.72	100	0	21.59	100	0	21.40	100	0	20.60	100
1	22.46	99.1	1	5.11	23.6	1	12.68	59.3	1	14.63[b]	71.2
2	16.33	72.0	2	1.06	4.92	2	8.00	37.4	2	11.18[b]	54.3
3	9.56	42.2	3	0.10	0.46	3	4.68	21.9	3	7.65	37.6
4	5.55	24.4	4	0.00	0.00	4	2.77	12.93	4	5.50[b]	26.7
6	1.61	7.09	..	...	...	6	0.70	3.27	6	2.55[b]	13.6
7	1.12	4.94	..	...	...	7	0.38	1.78	7	1.79	8.68
8	0.84	3.20	..	...	...	8	0.06	0.28	8	1.29	6.26
10	0.37	1.63	..	...	...	10	0.00	0.00	10	0.51	2.36
11	0.32	1.41	..	...	...	..	...	...	11	0.30	1.46
13	0.20	0.88	..	...	...	..	...	...	13	0.00	0.00
30% Water			40% Water			50% Water			60% Water		
0	20.71	100	0	20.18	100	0	19.85	100	0	19.39	100
1	16.31[b]	78.8	1	16.88[b]	83.7	1	17.68[b]	89.0	1	17.75	91.6
2	13.62	65.8	2	15.04	74.7	2	16.43[b]	82.8	2	16.80[b]	86.7
3	10.87	52.4	3	13.56	67.2	3	15.14	76.3	3	15.79[b]	81.4
4	8.98	43.4	4	11.68[b]	57.8	4	13.99	70.5	4	14.85[b]	76.7
6	4.79	23.2	6	8.16	40.5	6	11.21[b]	56.5	6	13.10[b]	67.6
7	3.59	17.4	7	6.76	33.6	7	10.20[b]	51.4	7	12.13[b]	62.7
8	2.45	11.8	8	5.91	29.4	8	9.17	46.2	8	11.37	58.6
10	0.99	4.77	10	4.27	21.2	10	7.67	38.6	10	9.90	51.1
11	0.42	2.03	11	3.80[b]	18.8	11	6.82	33.4	11	9.43[b]	48.6
13	0.00	0.00	13	2.51	12.5	13	5.49[b]	27.6	13	7.93[b]	40.8
70% Water			80% Water			90% Water			100% Water		
0	18.97	100	0	18.52	100	0	18.09	100	0	17.91	100
1	17.71[b]	93.5	1	17.22	93.2	1	16.60	91.8	1	5.78	32.3
2	16.94	89.5	2	16.21[b]	87.6	2	15.33[b]	84.9	2	0.91	5.08
3	16.12	85.2	3	15.01[b]	81.1	3	14.15[b]	78.3	3	0.70	3.91
4	15.55[b]	81.8	4	14.29	77.2	4	12.98	71.8	4	0.55	3.07
6	13.81[b]	72.8	6	12.34[b]	66.7	6	10.31[b]	57.1	6	0.30[b]	1.68
7	13.07[b]	69.4	7	11.53[b]	62.3	7	9.38	51.8	7	0.27	1.66
8	12.34	65.2	8	10.68[b]	57.6	8	7.99[b]	44.3	8	0.20	1.12
10	11.04	58.3	10	9.35	50.5	10	6.37[b]	35.2	10	0.15	0.84
11	10.20[b]	53.8	11	8.59[b]	46.3	11	5.65	31.3	11	0.13	0.73
13	8.50[b]	45.2	13	6.89	40.8	13	4.23[b]	23.4	13	0.09	0.50

[a] Percentage of water added in solvent.
[b] Color returns to titrated solution after initial discharge of blue iodine-starch color.

TABLE II.—EFFECT OF ADDED WATER ON THE STABILITY AT 50° OF H_2O_2-OXINE-GLYCEROL SOLUTIONS

Anhydrous			"As Is"			10% Water[a]			20% Water		
Days	Thio., Ml.	Resid. Perox., %	Days	Thio., Ml.	Resid. Perox., %	Days	Thio., Ml.	Resid. Perox., %	Days	Thio., Ml.	Resid. Perox., %
0	22.98	100	0	22.51	100	0	21.88	100	0	21.35	100
1	22.86	99.6	1	22.13	98.3	1	21.64	99.4	1	21.17	99.2
2	22.86	99.6	2	22.02	97.9	2	21.52	98.3	2	21.00	98.4
3	22.82	99.5	3	21.91	97.4	3	21.41	97.8	3	21.01	98.4
5	21.70?	94.5?	5	21.45	95.6	5	21.18	96.6	5	20.95	98.2
10	22.57	98.2	10	21.04	93.5	10	20.99	95.8	10	20.77	97.3
154	18.11	78.7	154	11.55	51.4	154	14.57	66.5	154	16.73	78.3
30% Water			40% Water			50% Water			60% Water		
0	20.82	100	0	20.49	100	0	19.98	100	0	19.65	100
1	20.68	99.4	1	20.28	99.0	1	19.78	99.0	1	19.49	99.2
2	20.56	98.6	2	20.35	98.4	2	19.78	99.0	2	19.39	98.6
3	20.53	98.5	3	20.16	98.3	3	19.69	98.5	3	19.36	98.4
5	20.51	98.4	5	20.10	98.1	5	19.77	98.8	5	18.36?	93.4?
10	20.33	97.7	10	20.07	98.0	10	19.62	98.3	10	19.29	98.2
154	15.19	73.8	154	14.01	68.4	154	15.98	79.7	154	14.60	74.4
70% Water			80% Water			90% Water			100% Water		
0	19.14	100	0	18.67	100	0	18.51	100	0	18.10	100
1	18.97	99.2	1	18.51	99.3	1	18.40	99.4	1	18.09	99.9
2	18.97	99.2	2	18.48	99.0	2	18.36	99.2	2	18.00	99.5
3	18.93	99.0	3	18.46	98.9	3	18.34	99.1	3	18.00	99.5
4	18.92	99.0	4	18.47	98.9	4	18.33	99.1	4	18.02	99.5
6	...	...	6	18.38	98.5	6	18.29	98.7	6	17.97	99.2
10	18.87	98.5	10	18.44	98.8	10	18.25	98.5	10	18.00	99.5
154	16.74	87.4	154	17.54	94.0	154	17.16	92.5	154	24.62	136[b]

[a] Water added to solvent.
[b] Increased concentration due to evaporation. Another lot (at 50°) showed 62.3% after 222 days.

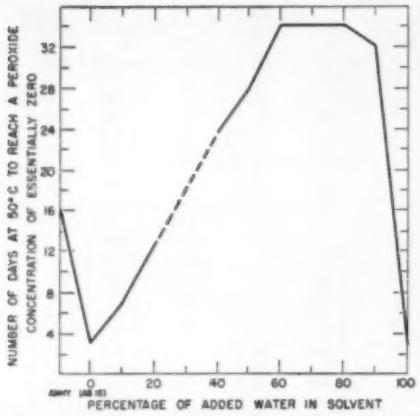

Fig. 2.—Effect of added water on stability of hydrogen peroxide in glycerol with and without oxine at 50°.

added water content of 70%. This is similar to the case of urea peroxide-glycerol solutions, with varying amounts of added water, where a maximum stability was attained in the region between 80 and 90% added water. In that case, however, the anhydrous solution was more stable than any of the others.

The solution with oxine shows an increasing stability with decreasing amounts of glycerol (increasing amounts of water). These solutions, as was expected, are relatively stable at 50° for a considerable length of time.

After storage for some time, the peroxide-glycerol solutions, without oxine, show a return of color of starch indicator after the initial titration is completed, and the titrated solution is allowed to stand. This is not due to under-titration, since it is shown by solutions to which an extra drop of thiosulfate has been added, nor is it due to the normal oxidation of iodide by air, since a control iodide solution shows no oxidation for almost half an hour. If the color thus formed is discharged with more thiosulfate, the procedure may be repeated until more than the original amount of thiosulfate has been used in this secondary titration. This renewal of color is not observed with solutions which are stabilized with oxine. The notation in Tables I and II of the return of color is not consistent, since the change may not be noticed if the samples being titrated are the last of a set, and are discarded immediately.

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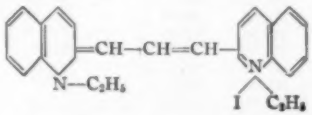
The Bactericidal and Bacteriostatic Effects of Pinacyanole*

By A. BACHEM†

Staining properties of pinacyanole on bacteria, its *in vitro* germicidal effect, its toxicity, and certain other characteristics have been determined and the results are reported in this paper. The conclusion reached as a result of these studies is that pinacyanole may possess useful therapeutic properties.

EXPERIMENTAL WORK on photosensitization of bacteria through photographically used sensitizers has led to the accidental discovery of strong bactericidal and bacteriostatic effects of one of these substances. The substance was pinacyanole, Eastman Kodak 622, or carbocyanine, a quinoline derivative, $C_{25}H_{25}N_2I$.

According to Mills and Hamer (1) the most probable structure is



Three other cyanine dyes with a chemically similar structure and with photographically similar properties (namely, dicyanine, cryptocyanine and neocyanine) showed no particular germicidal activity. A particular reason for the high germicidal activity of pinacyanole in comparison with the inefficiency of the other cyanines was not evident.

* Received November 1, 1950, from the Department of Physiology, University of Illinois, Chicago Professional Colleges.

† The early experimental work was conducted in collaboration with E. R. Kirch, Associate Professor of Chemistry, and M. Feiler, who was temporarily associated with the Department of Bacteriology of the University of Illinois, Chicago Professional Colleges.

SOLUBILITY

Pinacyanole was found to be soluble in water at room temperature about 1:2,500, and in moderately heated water about 1:1,000. Microscopical precipitation occurred after about three weeks. The watery solution appeared to be violet-blue.

The dye dissolved in 96% alcohol at room temperature 1:1,000, with a blue color.

It dissolved in a 20% glucose solution 1:500. The liquid became thick and gelatinous after a few hours; it was easily made fluid again by moderate heating and it remained fluid at body temperature.

It dissolved in a 5% glycerol solution about 1:1,000 with the same behavior as noted above. It dissolved most readily in a solution containing 20% glucose and 5% glycerol, with similar characteristics. It was soluble in human blood serum at body temperature 1:2,000; when added to human blood in diluted solutions, no precipitation occurred. It precipitated, however, upon the addition of sodium chloride. The addition of 5–15% of blood serum to mixtures containing sodium chloride diminished the precipitation greatly. It was also soluble in bile to a considerable degree.

VITAL STAINING

Bacteria were readily stained by pinacyanole, if suspended in the dye solution, as observed under the microscope by the hanging-drop method. Vital staining of diphtheria bacilli occurred in concentrations as low as 1:20,000. The rapidity of staining depended largely upon the concentration of the dye.

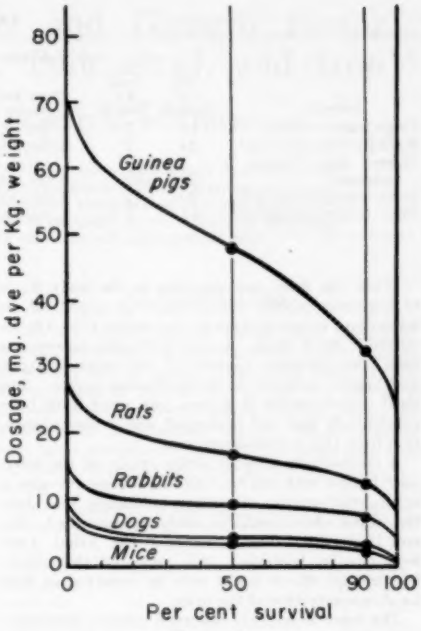


Figure 1

GERMICIDAL EFFECT *IN VITRO*

The bacteriostatic and bactericidal actions of pinacyanole were studied in test tube experiments. The pinacyanole was added to, and mixed with the bacterial broth cultures as a glucose solution of varying strength. The results obtained are shown in Table I.

The standard determination of the phenol coefficient (for *Staph. aureus*) gave a figure of 13.3 for pinacyanole.

TOXICITY

The toxicity of pinacyanole was studied on the following experimental animals: dogs, rabbits, guinea pigs, rats, and mice. Intravenous injections were given to dogs and rabbits; guinea pigs were injected subcutaneously; rats and mice intraperitoneally. The survival percentage was plotted against the dosage in terms of mg. pinacyanole per Kg. body weight. The 50% and 90% survival figures were read off the curve (see Fig. 1). The former were considered as lethal dose, the latter as tolerance dose. The two doses were also given in terms of approximate concentration of the drug in the body fluids after complete absorption and before excretion. (Total water was 65% of body weight.) This theoretical figure was never reached, especially in the case of subcutaneous injection. A high concentration in blood must have occurred through intravenous injection. This concentration was figured for dogs and rabbits on the basis of 7.2 and 5% of body weight, respectively. Table II gives the toxicity figures obtained.

TABLE I

Organism	Bacteriostatic Dose	Bactericidal Dose, Min. 1	2–2½	5	15–20	30
Diphtheria bacillus	1:1,000,000	1:5,000	1:10,000	....	1:40,000	1:80,000
Streptococcus hemolyticus	1:150,000	...	....	1:10,000	1:20,000	....
Staphylococcus aureus	1:400,000	...	....	....	....	1:5,000
Typhoid bacillus	1:25,000	...	....	....	....	....
Pneumococcus						
Type I	1:200,000	...	....	1:5,000	....	....
Type III	.....	...	....	1:15,000	....	....
Gonococcus	1:3,200,000	...	....	....	....	....
Bacterium Welchii	1:400,000	...	....	....	....	....
Staphylococcus aureus[a]	1:330,000	...	....	1:2,000	....	....

[a] Experiment kindly conducted by H. W. Cromwell of the Abbott Laboratories, with horse serum at body temperature.

TABLE II

Animals	No. of Animals	Tolerance Dose: Mg. Drug/Kg. Weight	Tolerance Dose: Drug/Body Fluids	Tolerance Dose: Drug/Blood	Lethal Dose (LD_{50}): Mg. Drug/Kg. Weight	Lethal Dose (LD_{50}): Drug/Body Fluids	Lethal Dose (LD_{50}): Drug/Blood
Dogs (intravenous)	15	2.7	1/240,000	1/27,000	3.7	1/170,000	1/20,000
Rabbits (intravenous)	24	7	1/90,000	1/7,000	9	1/70,000	1/5,500
Guinea pigs (subcutaneous)	20	32	1/20,000	....	47	1/14,000	....
Rats (intraperitoneal)	30	12	1/50,000	....	16	1/40,000	....
Mice (intraperitoneal)	44	1.5	1/400,000	....	3	1/200,000	....

While the final concentration in the body fluids of dogs and rabbits was of the order 1/100,000, an immediate concentration of the order 1/10,000 occurred in their blood. In rats and mice, however, a high concentration, perhaps of the order 1/10,000 and higher, occurred in the peritoneal cavity. The local concentration in guinea pigs must have been particularly high and prolonged, due to slow absorption from the subcutaneous area.

A comparison, though rather crude, of the tolerance figures with the bacteriostatic doses revealed a substantial margin of safety, indicating that bacteriostatic effects could be safely accomplished. Direct bactericidal doses resembled the lethal doses very closely, however; this indicated that direct bactericidal effects could only be expected at high local concentration of the drug.

The most frequently observed effects after injection, depending upon dosage, method of injection, and animal species, were: (*a*) immediate convulsions, followed by death; (*b*) mild convulsion and recovery; (*c*) restless or depressed appearance; (*d*) death, immediate, or after hours or days, or survival; (*e*) often hemolysis was present; (*f*) necropsy revealed very blue kidneys, blue spleen liver, and intestines, and a somewhat bluish appearance of most organs; (*g*) necrosis of the liver, degenerative changes in the stomach wall, and necrosis of the cutaneous injection area were observed once; (*h*) blood plasma and urine appeared bluish for several hours or days; (*i*) histological studies of the various organs were not attempted.

THERAPEUTIC AND PROPHYLACTIC EXPERIMENTS

Experiments were conducted on guinea pigs, inoculated with cultures of diphtheria bacilli, and on mice, inoculated with *Streptococcus hemolyticus*. A comparison of the unprotected controls with animals protected by the drug revealed prolongation of life or even survival of the latter ones.

Four guinea pigs were inoculated with lethal doses of diphtheria bacilli subcutaneously. Just before and after inoculation the wound was treated with an aqueous suspension of pinacyanole 1:100 or 1:250. The wounds of four controls were treated in the same way with physiological saline. The controls died after two to four days, with characteristic necropsy signs. The wounds of the protected animals healed without necrosis.

Seven mice were inoculated with a minimal lethal dose of *Streptococcus hemolyticus*. Of these, five died after about one day; two were still alive after six weeks.

Nine mice, inoculated in the same way, were given 0.5 mg. of the dye per Kg. weight eighteen hours later. At that time several of the animals were in a toxic condition. Two of these animals died after three days; seven others were still alive after 6 weeks.

Although these experiments appeared promising for the prophylactic and therapeutic use of pinacyanole, they were not extended to other animals and bacteria.

SPECTRAL ABSORPTION CURVES

Pinacyanole, in an aqueous solution, had a characteristic absorption curve, showing 2 maxima at 553 and 603 mμ, separated by a minimum at 572 mμ. Except for a slight shift toward longer wave lengths, this absorption resembled the one of oxyhemoglobin, particularly if a pinacyanole solution 1/200,000 was compared with hemolyzed blood, diluted 1/300. By means of this characteristic absorption at low concentration, the dye could easily be identified in plasma, lymph, tissue fluids, urine, and feces. This distribution, retention, and secretion of the dye could well be studied spectroscopically. Such experiments were not conducted, however.

The substance fluoresced mildly in the near ultraviolet. This property may also serve in identification of the dye.

SUMMARY

Experiments on pinacyanole revealed a strong germicidal effect, promising clinical usefulness of the dye.

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Comparative Hemopoietic and Growth Factor Responses to Vitamin B_{12}, Folic Acid, and Iron*,†

By ERSILIA F. DE GUIA and MORRIS M. WOLFRED‡

Experimental data have been presented on the effectiveness of vitamin B_{12}, folic acid, and iron on anemia in chicks produced by a basal deficient diet. Hemopoietic and growth factor values for combinations of folic acid, vitamin B_{12}, and iron have been demonstrated in varying dosages and modes of administration. Results illustrate the rate of recovery, growth, and effectiveness of the supplements.

EXPERIMENTAL evidence has indicated a further need for testing the effectiveness of folic acid and vitamin B_{12} on anemic chicks, and for additional information concerning the hemopoietic value of combinations of folic acid and vitamin B_{12} with iron; no previous work has been presented using such combinations. In addition, attempts were made to correlate the growth factor of folic acid and vitamin B_{12} with iron.

Gibson and Howard (1), in 1923, demonstrated that a diet high in iron content produced a favorable influence in pernicious anemia. Elvehjem and Hart (2), in 1929, reported that day-old chicks placed on a diet of cow's milk together with polished rice, calcium carbonate, and sodium chloride became anemic. The addition of ferrous sulfate to the ration stimulated hemoglobin synthesis within a short period of time. Hogan and Parrott (3), in 1940, observed that day-old chicks fed a folic acid-deficient diet developed anemia and failed to grow. Nichol, Dietrich, Elvehjem, and Hart (4), in 1949, observed that a folic acid deficiency in chicks fed a purified ration was accentuated by supplying vitamin B_{12}. Nichol, Harper, and Elvehjem (5), in 1949, induced severe anemia in chicks by intramuscular injections of phenylhydrazine hydrochloride after a depletion period on a folic acid-deficient ration. Pure vitamin B_{12} completely replaced liver extract in stimulating the formation of hemoglobin in the presence of folic acid. Schaefer, Salmon, Strength, and Copeland (6), in 1950, reported that vitamin B_{12} could replace folic acid under their experimental conditions; however, slightly higher hemoglobin levels were consistently observed when both nutrients were present in the diet. Vitamin B_{12} has been shown to be a growth factor for rats (7), pigs (8), and chicks (9, 10).

* Received November 21, 1950, from the University of Southern California, School of Pharmacy, Department of Pharmacology.

† This paper is based on a thesis presented by Ersilia F. De Guia to the Graduate School of the University of Southern California, in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.

‡ Assistant Professor of Pharmacology, the University of Southern California, School of Pharmacy, Los Angeles.

EXPERIMENTAL

Day-old white Leghorn chicks with numbered rings on their legs for identification were placed in electrically heated cages. The chicks were given a special prepared diet deficient in folic acid and were allowed to indulge in the special feed and water *ad libitum* until they became anemic. Blood samples were taken periodically by wing vein punctures, and red blood cell counts and hemoglobin levels were recorded.

The folic acid-deficient basal ration consisted of the following formula:

Casein	25.0 Gm.
Cerelose	50.7 Gm.
Gelatin	10.0 Gm.
Soybean oil, *q. s.*	6.0 Gm.
Salts[1]	5.0 Gm.
Methionine	0.3 Gm.
Cod liver oil	2,000.0 mg.
Thiamine	0.4 mg.
Riboflavin	0.8 mg.
Nicotinic acid	5.0 mg.
Pyridoxine	0.6 mg.
Calcium pantothenate	2.0 mg.
Biotin	0.02 mg.
Choline chloride	200.0 mg.
Inositol	100.00 mg.
α-Tocopherol	0.30 mg.
Vitamin K_1	2.50 mg.

Blood counts and hemoglobin levels were taken at weekly intervals until the red blood cell count reached below two million/cu. mm. of blood and the hemoglobin level was 5 Gm. or less per 100 cc. of blood. This has been previously determined as the anemic level in chicks (3). Twenty-four to thirty days were usually required to reach the anemic level.

Of 180 chicks given a folic acid-deficient diet, 132 survived the diet to reach the anemic level, indicating a mortality of 26.6%. The chicks affected by the deficiency showed body tremors, extended wings with inability to maintain balance, and signs of paralysis. They remained in this condition two to three days before dying. All chicks showed poor feathering, poor growth, enlarged crops, soft stools, and anorexia.

Once anemic, the chicks were divided into five main groups according to their mode and manner of treatment. They were weighed at the peak of their anemia and their weights were recorded

[1] Salts used according to nutritional requirements, *J. Nutrition*, **32**, 460(1946).

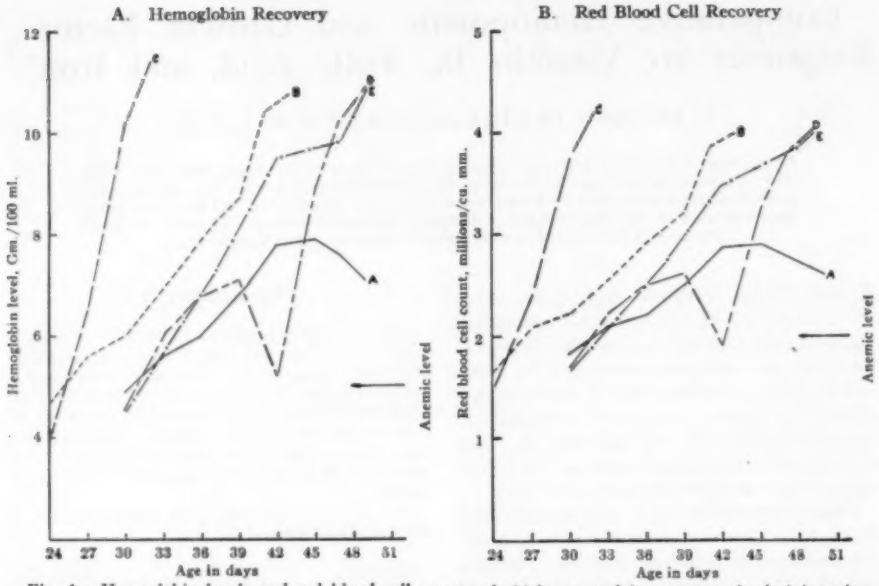

Fig. 1.—Hemoglobin levels and red blood cell counts of chicks treated by parenteral administration. (Doses: Vitamin B_{12}, 6 μg.; folic acid, 6 mg.; ferric ammonium citrate, 6 mg.)
A, 6 μg. vitamin B_{12}; *B*, 6 mg. folic acid; *C*, 6 μg. vitamin B_{12} plus 6 mg. folic acid; *D*, 6 μg. vitamin B_{12} plus 6 mg. ferric ammonium citrate; *E*, 6 mg. folic acid plus 6 mg. ferric ammonium citrate.

weekly during their process of treatment. On the basis of dosage and manner of administration, each main group was divided into four subgroups, using three chicks as a set of controls for each subgroup. Upon treatment, blood counts and hemoglobin levels were taken at one- to two-day intervals (see Fig. 1).

DISCUSSION

According to Doyle, Matthews, and Roberts (11), chicks in confinement have red cell counts ranging from 2.0 to 2.3 millions/cu. mm. Kelly and Dearstyne (12) have reported that the number of red blood cells in confined chicks ranged from 2.02 to 3.49 millions/cu. mm.; in the majority of analyses the counts fell between 2.27 and 3.01 millions/cu. mm. Cook (13) has presented counts made by three investigators, as well as by himself, and concluded that the average red cell count of 2.5 millions/cu. mm. was a normal for twenty-six-day old to eighty-six-day old chicks. However, in our experiments the normal number of red blood cells ranged from 3.73 to 4.71 millions/cu. mm. for week-old white Leghorn chicks. This was found to be higher than the red cell counts reported for older chicks in the above literature.

Cook (13) also found that the average normal hemoglobin level for twenty-six-day old to one-hundred-thirty-eight-day old chicks was 10.2 Gm./100 cc. of blood. A hemoglobin level higher than this value was obtained when casein was used as the principal source of protein in the diet. The results of our experiments demonstrate that the normal hemoglobin levels for seven-day old white Leghorn chicks ranged from 11.1 to 12.8 Gm./100 cc. of blood.

Vitamin B_{12}.—Vitamin B_{12} in 6- or 3-μg. doses administered either parenterally or orally did not cause a significant improvement on the red blood cell counts and hemoglobin levels of anemic chicks. Vitamin B_{12} given parenterally in doses of 6 μg. produced greater growth in the chicks than did 3 μg. of vitamin B_{12}/100 Gm. of diet. All the chicks which were given 3 μg. of vitamin B_{12}/100 Gm. of diet died within ten days after treatment was begun. According to Charkey, *et al.* (14), vitamin B_{12} functions by enhancing the utilization of the circulating amino acids for the building of fixed tissues and a deficiency of this vitamin will result in slow growth. No improvement in the feathering of the chicks was noted.

Folid Acid.—The chicks given daily doses of 6 mg. of folic acid parenterally showed rises in red blood cell counts and hemoglobin levels up to the twentieth day of treatment, but folic acid was not sufficient to cause a return to normal. Six milligrams of folic acid was found to be more active orally and parenterally than 3 mg. Chicks treated with folic acid showed a greater increase in weight than those which received vitamin B_{12} as the supplement, thus confirming the work of Williams, Nichol, and Elvehjem (15). Normal feathering developed within four days. All of the chicks survived.

Folid Acid Plus Vitamin B_{12}.—Chicks treated with a combination of folic acid and vitamin B_{12} showed the greatest increase in weight. Blood counts and hemoglobin levels of chicks treated with 6 μg. of vitamin B_{12} plus 6 mg. folic acid returned to

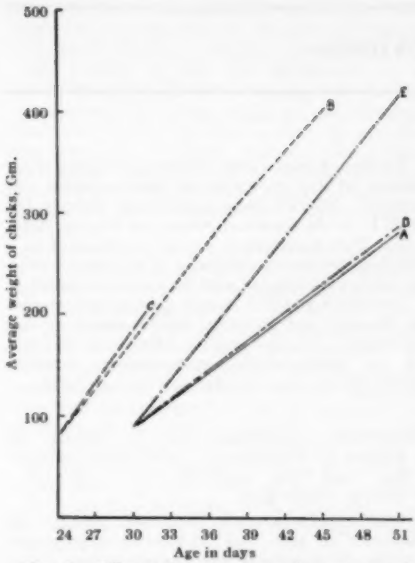

Fig. 2.—Effects of various supplements on the growth factor when administered parenterally. (Doses: vitamin B_{12}, 6 μg.; folic acid, 6 mg.; ferric ammonium citrate, 6 mg.)

A, 6 μg. vitamin B_{12}. *B*, 6 mg. folic acid; *C*, 6 μg. vitamin B_{12} plus 6 mg. folic acid; *D*, 6 μg. vitamin B_{12} plus 6 mg. ferric ammonium citrate; *E*, 6 mg. folic acid plus 6 mg. ferric ammonium citrate.

normal after nine daily injections. This dosage also produced the greatest increase in weight. Normal feathering began to develop within two days. Evidence indicates that vitamin B_{12} may function in an analogous manner to a catalyst by facilitating the uptake of folic acid by the tissues of the body, or vice versa.

Vitamin B_{12} Plus Iron.—Chicks treated with vitamin B_{12} plus iron showed slightly greater increases in weight than those treated with vitamin B_{12} alone. Three milligrams of ferric ammonium citrate plus 3 μg. of vitamin B_{12} given parenterally proved less active than 3 μg. of vitamin B_{12} administered alone in the same way. However, 6 μg. of vitamin B_{12} plus 6 mg. ferric ammonium citrate produced a hemopoietic response comparable to that produced by 6 mg. of folic acid plus 6 mg. of ferric ammonium citrate. This was also observed in the case of oral administration where ferrous gluconate was used instead of ferric ammonium citrate. No improvement in the feathering of chicks was noted.

Folid Acid Plus Iron.—Chicks treated with folic acid and iron parenterally or orally showed greater increases in weight than chicks treated with vitamin B_{12}, folic acid, and vitamin B_{12} plus iron. This combination also regenerated more red blood cells and higher hemoglobin levels than folic acid alone, but was not sufficient to bring them back to normal. Normal feathering began to develop within four days.

Controls.—Three chicks were used as controls for each supplement, and it was noted that the control chicks consumed less food than the treated anemic chicks; however, the increase in weight was not in proportion to the amount of food ingested. Chicks which received the supplements rapidly became more active than the controls.

SUMMARY

Of all the supplements administered, only the combination of folic acid and vitamin B_{12} actually cured the induced anemia, although other hemopoietic agents also influenced the regeneration of red blood cells and hemoglobin. Blood counts and hemoglobin levels returned to normal in twelve days following oral administration and in nine days following parenteral administration. Folic acid or vitamin B_{12} when combined with iron produced greater red blood cell and hemoglobin regenerations than either one used alone. In the decreasing order of their effectiveness on the growth factor, orally and parenterally, the supplements were found as follows: (*a*) folic acid plus vitamin B_{12} (*b*) folic acid plus iron, (*c*) folic acid, (*d*) vitamin B_{12} plus iron, and (*e*) vitamin B_{12}.

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Book Reviews

Austria-Codex 1950. 6th ed. By OTTO ZEKERT, WOLFDIETRICH WEIS, and HANS BRAUNER. Österreichischer Apotheker-Verlag, Vienna, 1950. 600 pp. 10.5 x 15 cm.

The sixth revised edition of the *Austria-Codex* was published under the auspices of the Ministry of Social Administration of Austria. The title implies that it is comparable to a pharmacopeia or formulary. Such, however, is not the case.

The *1950 Austria-Codex* is merely a register of specialty preparations distributed under trade mark names. In addition to the name of each preparation, the name and address of the manufacturer, the composition of the product, the dosage forms available, the indications, and directions for use are briefly stated. This little book is somewhat comparable in form and content to *Modern Drug Encyclopedia and Therapeutic Index*, except that the information is much more highly condensed. As a matter of fact, it is more highly condensed than in *Repertorium Pharmazeutischer Spezialpräparate*, the Swiss counterpart of the *Modern Drug Encyclopedia*. Like the two books with which it has been compared, a register of manufacturers is included and, in addition, the regulations of the Ministry of Social Administration concerning pharmaceutical specialties appear in an appendix.

The sixth edition of the *Austria-Codex* is a useful addition to the list of reference books employed in locating information relative to foreign specialties not distributed in the United States.

White Mineral Oil and Petrolatum. By ERICH MEYER. Chemical Publishing Co., Inc., Brooklyn, N. Y., 1950. vi + 135 pp. Illustrated. 14 x 22 cm. Price $4.75.

Although both petrolatum and liquid petrolatum are widely used pharmaceutical products, this use represents only a small proportion of the many applications of white mineral oil and petrolatum in other industries and processes. Despite the widespread use of these products, one does not find more than rather sketchy information concerning their chemical and physical characteristics and their uses in textbooks of pharmacy or chemistry. The author has prepared this book to supply information heretofore unavailable except by a rather extensive search of the literature. The data and information presented are based largely on industrial research and laboratory and factory experience in which the author has played a leading role. The constituents and nature of crude oil, its refining, the quality standards of white mineral oil and petrolatum, and the processes by which they are manufactured, are discussed in detail. A chapter on the applications of white mineral oil and petrolatum is unusually complete and presents an excellent survey of the many industries which depend upon these products for many of their processes and the production of their commodities.

Another chapter covers methods of testing white mineral oil and petrolatum for quality, purity, and identity. Most of these methods are those of the U. S. P. or the American Society for Testing Materials. This statement is not to be construed as a criticism, because the majority of the tests of these two agencies originated with the petroleum industry.

The fourth and final chapter presents information on shipping and handling white mineral oil and petrolatum. In this chapter information of interest to pharmaceutical manufacturers handling petrolatum products in large volume is included.

Heterocyclic Compounds. Vol. 2. Edited by ROBERT C. ELDERFIELD. John Wiley and Sons, Inc., New York, 1951. vii + 571 pp. 15 x 23.5 cm. Price $15.

Volume 2 of *Heterocyclic Compounds* considers the chemistry of five- and six-membered polycyclic compounds containing one oxygen or one sulfur atom. Thus, possibly for the first time, the large group of naturally occurring compounds including anthocyanins, flavones, flavanols, and comparable compounds have been critically and exhaustively covered in book form.

In order to present a maximum amount of information in a minimum of space, the details of the isolation and chemistry of a large number of natural products coming within the scope of this volume have been summarized in tables with adequate references. The critical discussion of the parent substances should, however, provide sufficient background to enable anyone interested in the subject to read and understand the details of published work on these naturally occurring substances.

The scope of the book can be readily appreciated from the chapter headings reproduced below:

1. Benzofuran and Its Derivatives
2. Isobenzofuran, Phthalan, and Phthalide
3. Dibenzofuran (Diphenylene Oxide)
4. Thionaphthene
5. Dibenzothiophene
6. Coumarins
7. Isocoumarins
8. Chromones, Flavones, and Isoflavones
9. Chromenols, Chromenes, and Benzopyrylium Salts: The Anthocyanins
10. Chromanones, Flavanones, Chromanols, and Flavanols: Catechin, Brazilin, and Hematoxylin
11. Chromans
12. Xanthones, Xanthenes, Xanthydrols, and Xanthylium Salts
13. Fluorans, Fluoresceins, and Rhodamines
14. Thiochromans and Related Compounds

The book is provided with an adequate index, is well documented, and the printing and binding are of an entirely satisfactory quality.

The Amino Acid Composition of Proteins and Foods. 2nd ed. By RICHARD J. BLOCK and DIANA BOLLING. Charles C Thomas, Springfield, Ill., 1951. xxxviii + 576 pp. Illustrated. 16 x 25.5 cm. Price $10.50.

In this enlarged second edition all new developments since 1945 in the field covered by the title have been made available. Included among these new developments are the use of biological methods employing microorganisms and specific enzyme systems for the routine estimation of all the known amino acids.

Quite detailed experimental directions are given for the determination of the 21 commonly accepted and 6 other amino acids in proteins and foods. In these directions gravimetric, colorimetric, gasometric, enzymatic, iodometric, polarographic, and selective oxidation methods are discussed. In nearly every instance alternative methods for the determination of each amino acid are presented along with the principle involved, the historical development, the reagents required, the experimental procedure, and the inherent errors of each method.

The book is documented with nearly 1,400 references, and its utility is enhanced by the judicious use of more than 200 tables. The index appears to be adequate, and the printing and binding are of good quality.

Perfumery Synthetics and Isolates. By PAUL Z. BEDOUKIAN. D. Van Nostrand Company, Inc., New York, 1951. viii + 488 pp. 15.5 x 24 cm. Price $7.

Extensive advances in the synthetic perfume industry have occurred during the past twenty-five years. Despite these advances, the field of synthetics and isolates used by the perfume industry has not been made the subject of books covering these materials exclusively and in a comprehensive manner. To be sure, many of these compounds have been described or briefly referred to in such books as the excellent series by Guenther on the essential oils and in the Terpenes, by Simonson, but these have covered only a part of the field.

In writing this book, the author has succeeded in making it informative and useful to the perfume and flavor industries, and at the same time of general interest to chemists. Anyone expecting it to be an academic treatise, however, will be disappointed, because it does not come in that category. On the other hand, it appears to be entirely adequate from a scientific standpoint and includes the history, chemistry, physical and chemical properties, manufacturing uses, and other pertinent data of the principal perfumery compounds. Not all synthetics used in the perfume industry are discussed in this volume, but the most important ones have been treated in an interesting and readable manner. The present volume should help fill the gaps in the literature on perfumery synthetics, and save anyone needing basic information and references to the original literature considerable time in making searches for methods of synthesis and other information on perfumery synthetics and isolates. A feature of especial interest is the reproduction of information on trade and commerce for every compound, where this information can be obtained from the U. S. Tariff Commission.

The book is well documented and it has an excellent index. The printing and binding are of good quality. The book can be recommended for use by anyone having an interest in any one of the ramifications of the field covered.

Adsorption and Chromatography. By HAROLD GOMES CASSIDY. Interscience Publishers, Inc., New York, 1951. xix + 360 pp. Illustrated. 15 x 23.5 cm. Price $7.

This book constitutes Volume V of a series of books published under the general heading of "Technique of Organic Chemistry." It is the aim of the authors and the publisher to contribute through this series to a better understanding and a more rational and effective application of the techniques employed in the laboratory for the investigation of organic compounds. The present Volume V appears to be up to the high standards established by the preceding volumes. A successful attempt has been made to describe and explain by discussion of principles, the several methods by which adsorption may be applied to organic chemistry for the separation of mixtures. To accomplish this, the author begins by explaining the nature of adsorption and of the phenomena connected with it. The chemical and physical properties of phases, interfaces, and adsorptives necessary to gain an insight into the nature of separation processes have been particularly emphasized. A detailed description and explanation of the various types of adsorption methods are clearly and concisely stated. In one chapter of the book a few simple experiments are described by means of which the reader may familiarize himself with various techniques. Chromatography, ion-exchange adsorption, and partition chromatography constitute three other chapters of the book, in which these subjects are adequately presented. Throughout, the book has been written from the point of view of the organic chemist. The book is replete with references, both general and to the original literature. It has a good index, and the printing and binding are of the quality one has become accustomed to expect in books made available by Interscience Publishers.

Amino Acids and Proteins. By DAVID M. GREENBERG. Charles C Thomas, Springfield, Ill., 1951. ix + 950 pp. Illustrated. 16 x 26 cm. Price $15.

In this book the methods employed in the isolation, establishment of purity, measurement of size and shape, determination of physical and chemical characteristics, methods for the analysis of component units and products, and evaluations of the functions and properties of biologically important proteins and protein derivatives are presented. It is the aim of the book to present this information in such a way that it may be used to acquaint students and investigators with the more general methods employed in the study of the proteins, and to give a summary of the current knowledge and advancements of the subject matter. No topics requiring mathematical treatment have been included.

Of pharmaceutical interest is the discussion of biologically important proteins and their properties, including the plasma proteins and the hormone proteins. Also of especial interest is the section on the

significance of ACTH, of insulin, and of the growth hormone. Some recent advancements in immunology are stressed, and tables of information on the chemical properties of the amino acids, of the molecular weights of proteins, and of the biological characteristics of amino acids and proteins add to the interest and value of this book.

Compiled and edited by David M. Greenberg, Professor of Biochemistry and Chairman of the Division, University of California School of Medicine, Berkeley, Calif., and 18 collaborators, each of whom contributed sections covering subjects of his particular specialty, the book is completely documented, possesses a good index, and the typography is particularly pleasing to the eye and is conducive to ease in reading.

Federal Food, Drug and Cosmetic Law Administrative Reports, 1907–1949. Commerce Clearing House, Inc., Chicago, 1951. xvi + 1446 pp. 17 x 25 cm. Price $22.50.

This is the first of a series of books to be published under the auspices of the Food Law Institute, Inc., which was organized in 1949 by leading food manufacturers, and from whom its main support is derived. The Institute has two major objectives the first of which is to prepare basic research compilations and studies of the Federal Food, Drug and Cosmetic Act. The second objective is to stimulate the interest of university law schools and other university schools in offering basic and advanced instruction in food, drug, and cosmetic laws. In order to accomplish these aims, plans have been formulated to provide for the publication of authoritative reference books to be known as the "Food Law Institute Series" which will, because of the law of the U. S. regulating foods, also cover drugs and cosmetics.

This book is an indexed compilation of all official administrative reports relating to Federal food, drug, and cosmetic laws covering the period between the first report in 1907 and the next to the last report in 1949. The original reports are available, but they are scattered in many different places and are not always easy to locate promptly in all libraries or elsewhere when needed. They are one of the principal sources of complete and official information concerning the administration of the original Food and Drug Act of 1906 and the 1938 Federal Food, Drug and Cosmetic Act. The value of the compilation is enhanced by an introduction by Paul B. Dunbar, which traces briefly the progress of Federal food and drug legislation and the revisions which have occurred during the past 45 years.

BOOKS RECEIVED

Textbook of Organic Chemistry. 3rd ed. By E. WERTHEIM. The Blakiston Company, Philadelphia, 1951. xii + 958 pp. Illustrated. 16 x 23.5 cm. Price $5.

Experimental Spectroscopy. 2nd ed. By RALPH A. SAWYER. Prentice-Hall, Inc., New York, 1951. x + 358 pp. Illustrated. 15.5 x 23.8 cm. Price $6.65.

Administrative Medicine. HAVEN EMERSON, Editor. Thomas Nelson and Sons, New York, 1951. xvi + 1007 pp. Illustrated. 19 x 25.5 cm. Price $10.

Optical Crystallography. 2nd ed. By ERNEST E. WAHLSTROM. John Wiley and Sons, Inc., New York, 1951. vii + 247 pp. Illustrated. 15.5 x 23.5 cm. Price $4.50.

Scoville's The Art of Compounding. 8th ed. By GLENN L. JENKINS, DON E. FRANCKE, EDWARD A. BRECHT, and GLEN J. SPERANDIO. The Blakiston Company, Philadelphia, 1951. vii + 515 pp. Illustrated. 18.5 x 26 cm. Price $7.50.

Addendum 1951 to the British Pharmacopoeia, 1948. Published under the direction of the General Medical Council. Constable and Co., Ltd., London, 1951. xviii + 114 pp. 14.5 x 23 cm. Price 17s. 6d.

Schimmel Briefs. Vol. I. Nos. 1–189, from April, 1935, to December, 1950. Schimmel and Co., Inc., New York, 1951. 195 pp. 22 x 28.3 cm.

Tablet Coating. By RONALD CLARKSON. Drug and Cosmetic Industry, New York, 1951. 75 pp. Illustrated. 14 x 21 cm. Price $4.

Accepted Dental Remedies. 16th ed. Council on Dental Therapeutics, American Dental Association, Chicago, 1951. xv + 207 pp. 16 x 20.5 cm. Price $1.50.

Medicinal Chemistry. Vol. I. C. M. SUTER, Editor-in-Chief. John Wiley and Sons, Inc., New York, 1951. vii + 473 pp. 15.5 x 23.5 cm. Price $12.

Fluoridation in the Prevention of Dental Caries. Council on Dental Health, American Dental Association, Chicago, 1951. 28 pp. 17.2 x 24.2 cm.

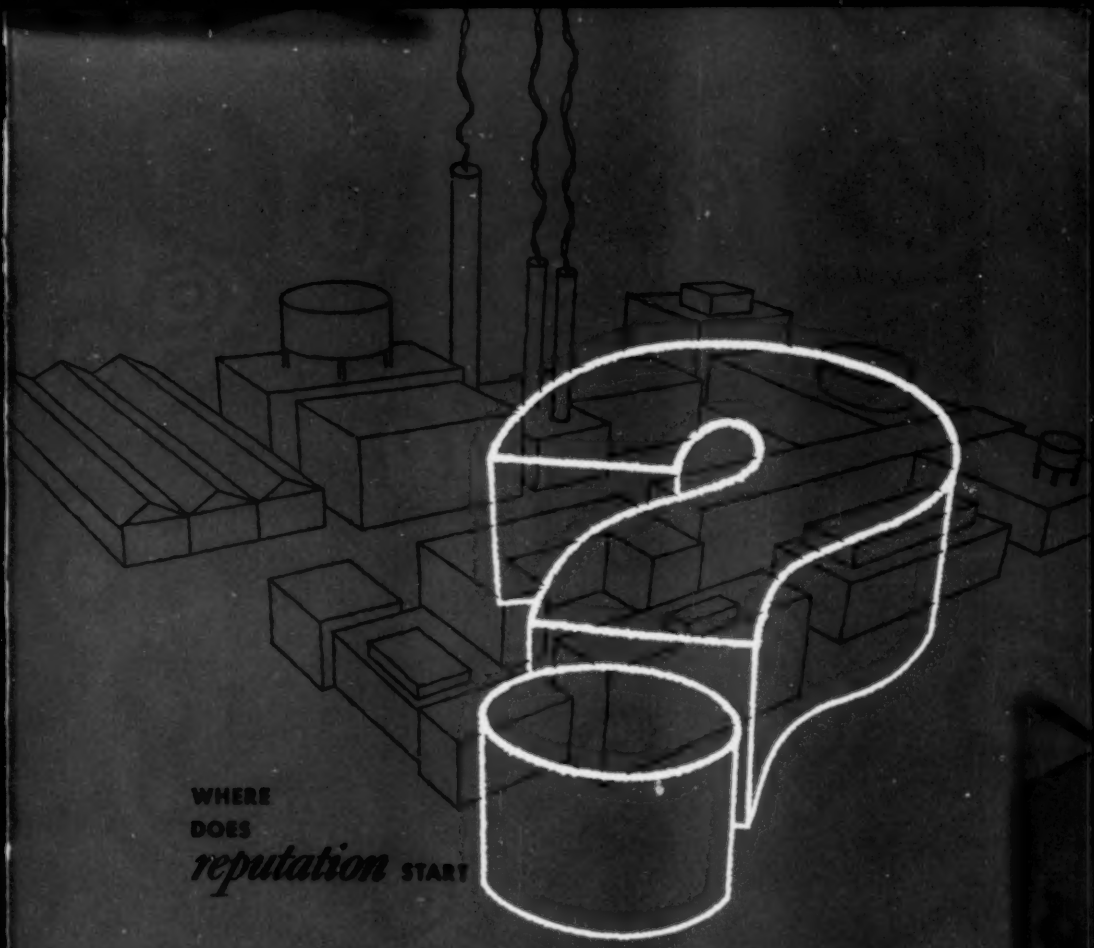
Paracelsus (Magic into Science). By HENRY M. PACHTER. Henry Schuman, Inc., New York, 1951. x + 360 pp. Illustrated. 14.5 x 21.8 cm. Price $4.

Geschichte der Pharmazie und Chemie. 3rd ed. By H. VALENTIN. Wissenschaftliche Verlagsgesellschaft m.b.H., Stuttgart, Germany, 1950. 128 pp. 17 x 23.8 cm. Price DM 7.50.

Business Practices Under Federal Antitrust Laws, 1951 Symposium. Proceedings of the Third Annual Meeting, Section on Antitrust Law, New York State Bar Association, January 24, 1951. Commerce Clearing House, Inc., Chicago, 1951. 190 pp. 15.3 x 22.8 cm. Price $2.

Active Carbon. By JOHN W. HASSLER. Chemical Publishing Co., Inc., Brooklyn, 1951. vi + 384 pp. 14.5 x 23.5 cm. Price $7.

1951 Pharmacy State Board Questions and Answers. By RALPH J. MILL. Clark and Wilson Company, Detroit, 1950. 94 pp. 22 x 28 cm.



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